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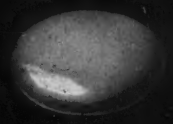

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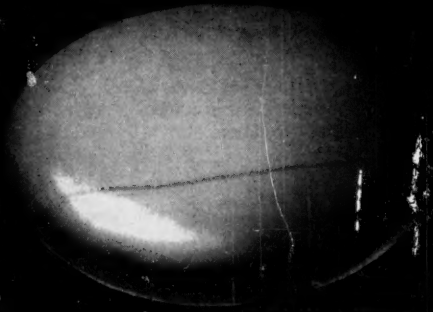

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LATENT PERIPHERAL ACTIONS	• FUNCTIONS INHIBITED BY EPINEPHRINE: INTESTINE ISOLATED • EPINEPHRINE VASODILATATION • VASOCONSTRICTION AND PRESSOR ACTION IN MAN CAUSED BY NICOTINE, VALSALVA MANEUVER, COLD, HYPERVENTILATION • STRIATED MUSCLE • METABOLIC EFFECTS OF EPINEPHRINE, I.E., INCREASE OF BMR, EPINEPHRINE HYPERGLYCEMIA • RENAL CORTEX ISCHEMIA FOLLOWING EPINEPHRINE, ASPHYXIA, ELECTRIC STIMULATION OF THE SPLANCHNIC NERVES, CRUSH SYNDROME	ADRENERGIC BLOCKADE NO BLOCKADE PREVENTION OR INHIBITION RELAXATION (LISSIVE) INHIBITION PREVENTION

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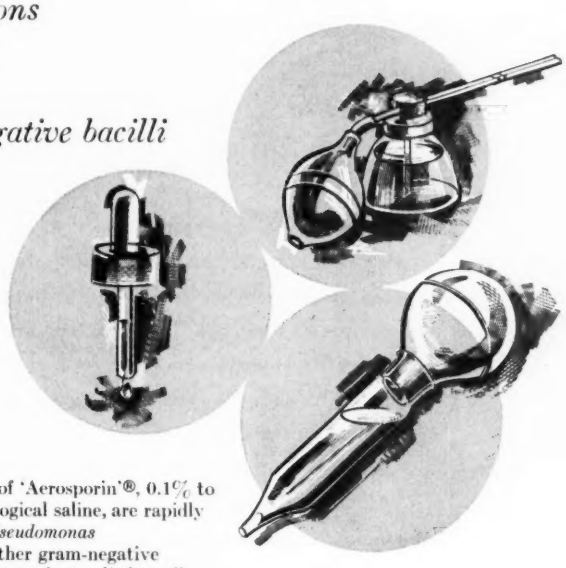

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THE INFLUENCE OF CORTISONE AND ADRENOCORTICOTROPHIC HORMONE ON BRUCELLOSIS. I. CORTISONE IN EXPERIMENTALLY INFECTED ANIMALS

By ROBERT ABERNATHY AND WESLEY W. SPINK

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(Submitted for publication April 21, 1952; accepted August 21, 1952)

A basic feature of the host-parasite relationship in brucellosis is the intracellular localization of Brucella in the tissues of animals and man (1–6). This intracellular parasitism is a contributing factor in the tendency of the disease to become chronic. Experimental studies have shown that intracellular Brucella organisms are afforded protection against antibiotics, and also against the lethal action of serum (7, 8). Brucella organisms in an extracellular position are readily destroyed by antibiotics, and by fresh serum. Prompt elimination of Brucella from the tissues of the host appears to depend, in part at least, upon dislodging the bacteria from their intracellular localization. The report on experimental tuberculosis by Hart and Rees (9) suggested that the host-parasite relationship in brucellosis could be disrupted by cortisone, and Brucella organisms would then be liberated from the cells of the host. The pathogenesis of brucellosis simulates that of tuberculosis in many respects. Of primary importance is the tendency of both tubercle bacilli and Brucella to localize in macrophages. Hart and Rees (9) demonstrated that cortisone enhanced both acute and chronic tuberculosis in mice. There was more dissemination of the infection in the cortisone-treated animals; the individual lesions showed more necrosis and caseation, and contained many more tubercle bacilli than were found in the non-treated control animals. Contrary to expectations, preliminary observations revealed that cortisone caused a marked proliferation and dissemination of Brucella organisms in animals with acute brucellosis, but the steroid did not provoke any demonstrable changes in the animals with chronic infections (10). The present studies also provided information for investigations on the influence of adrenocorticotropic hormone in human subjects having acute or chronic Brucella infections.

METHODS OF STUDY

The effect of cortisone on experimental acute and chronic brucellosis was studied in mice, guinea pigs, and rabbits. Infections were established with a representative strain of *Brucella abortus*, *Brucella suis*, and *Brucella melitensis*, all of which had been isolated from human sources. White male mice weighing approximately 20 gm. were used. The guinea pigs were all males and averaged 300 gm. in weight, while white male rabbits weighing around 2 kg. were studied. An animal was considered to have had an acute infection when but five to 15 days had elapsed from the time the bacteria were first introduced. A chronic infection was indicated when a month or more had elapsed. A suspension of cortisone, containing 25 mg. per ml., was injected intramuscularly into the thighs, alternating sides from day to day. Sterile physiologic saline solution was injected into control animals. Two different dosage schedules were employed in the animals with acute infections. Under the first schedule, cortisone was administered for three to five days prior to the establishment of infections, and then for five days to two weeks afterwards. Animals were pretreated with cortisone in a manner similar to that of Mogabgab and Thomas (11), who reported that rabbits were more susceptible to infections with group A hemolytic streptococci when pretreated with cortisone. The second schedule of cortisone therapy was started simultaneously with the infection and treatment carried out for two weeks. In the chronically infected animals, cortisone was administered for five days to two weeks.

At the completion of treatment in each group, all of the animals were sacrificed with chloroform. Forty-eight hours prior to death in the guinea pigs and rabbits Brucella antigen (Brucellergen) was introduced intradermally on the abdomen and evidence of a skin reaction ascertained at the time of sacrifice. Each guinea pig was given 0.15 ml. of Brucellergen and each rabbit 0.1 ml. of Brucellergen. Blood from the hearts was obtained for cultural purposes and for Brucella agglutination tests. Trypticase-soy broth [1] was used for blood cultures.

RESULTS

Influence of cortisone on the tissues of normal animals

When 0.25, 0.5, or 1.0 mg. of cortisone was injected daily into mice, each of these doses produced

[1] Baltimore Biological Laboratory, Baltimore, Maryland.

side effects such as decreased physical activity, ruffling of the fur, weight loss, and occasional diarrhea. The gross and microscopic changes in the livers, kidneys, and lungs were similar to those described by Antopol (12), and consisted of disseminated abscesses, which could be differentiated from the granulomatous reaction caused by Brucella. When a dose of 10 mg. of cortisone was administered daily to guinea pigs no side effects were elicited, and there were no abscesses noted in the tissues. A dose of 12.5 mg. daily of cortisone given to rabbits induced a marked lipemia (13). The administered hormone caused an enlarged and friable liver with a glossy appearance, and the hepatic cells appeared swollen with a clear cytoplasm and the nucleus situated at the periphery. In all three species, cortisone caused a reduction in the size of the spleens, which has been noted by others (14, 15). The adrenals were also reduced in weight.

Influence of cortisone on acute brucellosis

Mice. A group of mice were pretreated with 1 mg. of cortisone daily for five days and then injected intravenously with approximately 100 million *Br. suis* organisms. Suitable control mice were also observed as seen in Table I, A. It was intended to continue the daily injection of cortisone for two weeks after the animals had been infected, but this dose was too toxic, and the animals died before this period of time. Approximately the same numbers of Brucella were recovered from the livers and spleens of the cortisone-treated group as from the saline-treated animals. Gram-negative coliform organisms were cultured from the livers and spleens of the uninfected corti-

TABLE I

The influence of cortisone on acute brucellosis in mice

A

Animals pretreated with 1 mg. of cortisone daily for 5 days before being infected with *Brucella suis* and then treated for 5 more days after being infected. A control infected group of mice was similarly treated daily with 0.04 ml. of sterile physiologic saline solution instead of cortisone. Another control group, but uninfected, was treated with cortisone.

Group	Number of animals	Average days of treatment	Average total days of infection	No. of animals with Brucella in heart's blood at time of death	Average wt. of spleens, grams	No. of animals with Brucella cultured from		No. of microscopic lesions per 10 low-power fields in			
						Liver	Spleen	Liver	Spleen	Kidney	Lung
Infected—Cortisone	20	10	5	0/0	0.055	7/20	8/20	195	0	0	0
Infected—Saline	20	19	15	6/10	0.403	7/20	11/20	9	0	0	0
Uninfected—Cortisone	9	14	0	0/0	0.050	0/5	0/5	3	0	0	0

B

Animals pretreated with 0.5 mg. of cortisone daily for 3 days before being infected with *Br. suis* and then treated for 7 more days after being infected. A control infected group of mice was similarly treated daily with 0.02 ml. of saline solution instead of cortisone. Another control group, but uninfected, was treated with cortisone.

Infected—Cortisone	26	10	7	7/7	0.132	8/22	8/22	87	0	0	2/26
Infected—Saline	9	11	8	7/7	0.368	7/9	7/9	13	0	0	0/9
Uninfected—Cortisone	4	10	0	0	—	0/4	0/4	0	0	0	0/4

C

Animals splenectomized one year previously; pretreated with 0.5 mg. of cortisone daily for 5 days before being infected with *Br. suis* and then treated for 6 days more after being infected. A control infected group was similarly treated daily with 0.02 ml. of saline solution instead of cortisone.

Infected—Cortisone	5	11	6	4/4	—	4/5	—	110	—	1	6
Infected—Saline	4	11	6	4/4	—	4/4	—	27	—	10	8

D

Treatment with cortisone started at the same time that animals were infected with *Br. suis*. One mg. of cortisone was given daily for average of 12 days; a control group of infected animals received 0.04 ml. of saline solution.

Infected—Cortisone	10	12	12	0/1	0.069	4/10	5/10	74	0	0	0
Infected—Saline	8	14	14	5/7	0.267	2/8	7/8	16	0	0	0

sone-treated control animals that died. Gross abscesses were noted in the livers, spleens, and lungs of all the animals treated with cortisone. Brucella were recovered from only the livers and spleens. The outstanding observation in this experiment was the appearance of the livers in the Brucella-infected mice that had received cortisone; they were riddled with abscesses, many displaying such a degree of necrosis that structural detail was obliterated. The pronounced destruction of the hepatic tissue caused by cortisone was in marked contrast to the benign type of lesion induced by the injection of saline solution in which an occasional cellular infiltrate occurred without necrosis. The contrasting appearance of the hepatic lesions in the saline-treated animals and the cortisone-treated animals can be seen by comparing Figures 1 and 2. Giemsa-stained preparations of the livers from the cortisone-treated group revealed a striking appearance. The lesions appeared to be teeming with massive numbers of small, gram-negative, cocco-bacillary organisms, arranged in clumps, and giving the typical appearance of Brucella. That these organisms were Brucella was proved by isolation on agar media. The cytoplasm of the hepatic cells was packed with organisms. Many Kupfer cells also contained similar bacteria. In the livers from several animals, there were clumps of organisms in the hepatic sinusoids with no apparent cellular reaction around them. In the saline-treated infected animals, only rare bacteria were seen, and then only after considerable searching. When bacteria were present they were observed in the individual lesions, and none were seen in the parenchymal cells. In the uninfected cortisone-treated controls, bacteria were seen only in the abscesses. There was no essential difference noted in the gross and microscopic appearance of the spleens from the Brucella-infected mice treated with cortisone or with saline solution. The extensive hepatic involvement was absent.

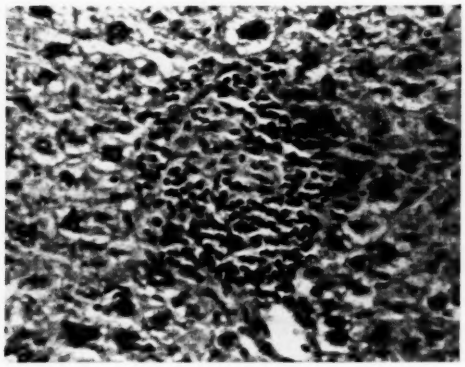

FIG. 1. FOCAL INFLAMMATORY LESION WITHOUT NECROSIS IN THE LIVER OF A MOUSE INFECTED WITH *Br. suis* AND PRETREATED WITH PHYSIOLOGIC SALINE SOLUTION

Hematoxylin and eosin, 300 ×.

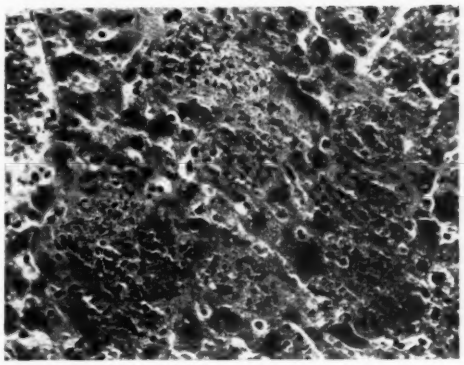

FIG. 2. MASSIVE INFLAMMATORY REACTION AND NECROSIS OF HEPATIC LESIONS OF MOUSE PRETREATED WITH 1 MG. OF CORTISONE DAILY FOR FIVE DAYS AND THEN INFECTED WITH *Br. suis*

Hematoxylin and eosin, 300 ×.

Because a pretreatment dose of 1 mg. of cortisone daily for five days proved to be too large, smaller doses of 0.5 and 0.25 mg. were administered daily, but even these doses proved too large since the animals died before the expiration of the two weeks' period of treatment. It was then decided to reduce the pretreatment time from five to three days in a group of mice infected with Brucella, giving 0.5 mg. of cortisone daily for three days. This schedule also resulted in the premature deaths of the animals. The data of this experiment are presented in Table I, B. *Br. suis* was recovered from the heart's blood of all the sacrificed, infected animals. It is to be noted that the spleens of the cortisone-treated animals were three times smaller than those that had received saline solution, but the spleens of the animals that had received 0.5 mg. of cortisone were twice as large as the spleens of the mice given 1 mg. of cortisone daily. Abscesses were noted in the spleens and livers of the

TABLE II

The influence of cortisone on acute brucellosis in guinea pigs

A

Three animals pretreated with 10 mg. of cortisone daily for 5 days, and then one infected with *Br. melitensis*, one with *Br. suis*, and one with *Br. abortus*. Treatment continued with cortisone for 5 more days. Three similarly infected animals treated in same way with saline solution.

Group	Number of animals	Average days of treatment	Average total days of infection	No. of animals with Brucella in heart's blood at time of death	Average wt. of spleens, grams	No. of animals with Brucella cultured from		No. of microscopic lesions per 10 low-power fields in			
						Liver	Spleen	Liver	Spleen	Kidney	Lung
Infected—Cortisone	3	10	5	0/3	0.92	2/3	3/3	7	0	0	0
Infected—Saline	3	10	5	0/3	0.88	2/3	3/3	1	0	0	0

B

Animals pretreated with 10 mg. of cortisone daily for 5 days, infected with *Br. suis*, and then treated for an average of 11 days more. A control group of infected animals was treated in similar manner with daily injections of saline solution.

Infected—Cortisone	5	16	11	3/3	1.09	4/5	4/5	4	1	0	—
Infected—Saline	5	19	14	3/5	2.80	5/5	5/5	9	13	0	—

cortisone-treated group, and many more colonies of *Br. suis* were recovered from the organs of these animals than from the saline-treated group. The extent of liver injury was less in the group of animals receiving 0.5 mg. of cortisone daily than in those treated with 1 mg. daily. Though there was more cellular infiltration in the cortisone-treated than in the saline-treated group, extensive hepatic necrosis and marked reproduction of brucella cells were absent.

Since the administration of cortisone to mice was uniformly associated with a marked diminution in the size of the spleen, this gave rise to speculation concerning the effect of cortisone on the course of brucellosis in splenectomized mice. Nine healthy mice splenectomized more than a year previously were available for study. Five were pretreated for five days with 0.5 mg. of cortisone daily, and four received saline solution. After infection with *Br. suis*, treatment was continued for six more days and the animals were sacrificed. The results are presented in Table I, C. Brucella organisms were recovered from the heart's blood of all nine animals, and more colonies were isolated from the livers of the cortisone-treated animals. Microscopically, the liver, kidneys, and lungs from all the splenectomized mice showed much more extensive involvement by Brucella than has been described elsewhere for intact animals (16). While the livers of the splenectomized animals treated with cortisone displayed more lesions, there was no essential difference in the histologic appearance of the two groups. There was an absence of highly destructive and necrotic hepatic lesions, and lack of evidence of marked proliferation of the Brucella organisms.

In all the preceding experiments, mice were treated with cortisone before infecting them with Brucella. Observations were made with a group of animals in which the institution of an infection and treatment with cortisone or saline solution were initiated simultaneously. Infected mice received 1 mg. of cortisone daily for a mean survival time of 12 days. The results are presented in Table I, D. More colonies of Brucella were cultured on agar plates from the livers of the cortisone-treated animals than from the saline-treated controls. In addition, more extensive involvement of the livers was noted in the cortisone-treated group, but the hepatic necrosis, evidence of suppuration and proliferation of Brucella organisms, was not as pronounced as in the animals that had been pretreated with cortisone.

Guinea pigs. Braude (17) had previously demonstrated in this laboratory that each of the three species of Brucella produced a different reaction in guinea pigs. Therefore, three pairs of guinea pigs were treated in the following manner: one animal in each pair was pretreated with 10 mg. of cortisone daily for five days, and then each of these animals was infected intraperitoneally with *Br. melitensis*, *Br. suis*, and *Br. abortus*, respectively, and treatment continued for an additional five days. Three control animals were handled in a

similar way except that saline solution was given instead of cortisone. The results are presented in Table II, A. No agglutinins were demonstrated in the blood of any of the animals. Although cultures of the heart's blood remained sterile, brucella organisms were recovered from the livers and spleens with more colonies being isolated from the cortisone-treated group. There was no essential difference in the brucella lesions noted in the two groups, except in two animals infected with *Br. suis*. In the livers of the saline-treated animals, small granulomas without necrosis were observed, whereas in the cortisone-treated animals many more lesions were seen and necrosis was apparent.

A larger number of guinea pigs were then pretreated with cortisone and infected with *Br. suis*. The results are found in Table II, B. The skin of none of the animals reacted to the intradermal injection of Brucella antigen. Brucella organisms were recovered from the heart's blood of the three surviving cortisone-treated animals, and from three of five saline-treated guinea pigs. Agglutinins were present in all the animals, there being no difference between the two groups. The spleens of the cortisone-treated animals weighed considerably less than the saline-control group. Brucella cells were uniformly isolated from the organs of all the animals sacrificed. More colonies were found in cultures from the cortisone-treated group. Cultures of the organs of those animals that died were overgrown with coliform bacteria. Microscopically, the livers and spleens revealed no difference between the two groups of animals in the number, size, and type of lesions and there was no difference in the number of organisms.

Rabbits. In an exploratory type of experiment with each of the three species of Brucella, similar to that carried out with guinea pigs, one in each of three pairs of rabbits was pretreated with 12.5 mg. of cortisone daily for five days, infected, and then treated for an additional five days. The other infected animal in each pair was treated with saline solution. The results are summarized in Table III, A. The intradermal reaction to Brucella antigen was positive in the three saline-treated rabbits, but only slightly positive in one of the cortisone-treated animals and absent in the other two. There was also a significant reduction in the agglutinin titer of the cortisone-treated animals compared to those receiving saline solution. The weight of the spleens was markedly reduced in the cortisone-treated group. Cultures of the organs from all the rabbits yielded Brucella, with more colonies from the cortisone-treated animals. The only significant changes in the tissues occurred in the livers. In the saline-treated group, there were small granulomas without necrosis, but in the cortisone-treated rabbits the number of lesions was not only increased, but considerable necrosis of the hepatic cells with an infiltrate of polymorphonuclear neutrophiles was apparent (Figure 3). Bacteria were seen only in the hepatic lesions of the

TABLE III

The influence of cortisone on acute brucellosis in rabbits

A

Three animals pretreated with 12.5 mg. of cortisone daily for 5 days, and then one infected with *Br. melitensis*, one with *Br. suis*, and one with *Br. abortus*. Treatment with cortisone was continued for 5 days more. Three similarly infected animals were treated in same way with saline solution.

Group	Number of animals	Average days of treatment	Average total days of infection	No. of animals with Brucella in heart's blood at time of death	Average wt. of spleens, grams	No. of animals with Brucella cultured from		No. of microscopic lesions per 10 low-power fields in			
						Liver	Spleen	Liver	Spleen	Kidney	Lung
Infected—Cortisone	3	10	5	3/3	1.13	3/3	3/3	53	0	—	—
Infected—Saline	3	10	5	3/3	2.15	3/3	3/3	9	0	—	—

B

Animals pretreated with 12.5 mg. of cortisone daily for 5 days, infected with *Br. suis*, and then treated with cortisone for 14 days more. Infected control group was treated in similar manner with saline solution.

Infected—Cortisone	5	18	13	4/4	1.55	4/4	4/4	27	48	0	—
Infected—Saline	5	19	14	5/5	8.12	3/5	4/5	23	40	0	—

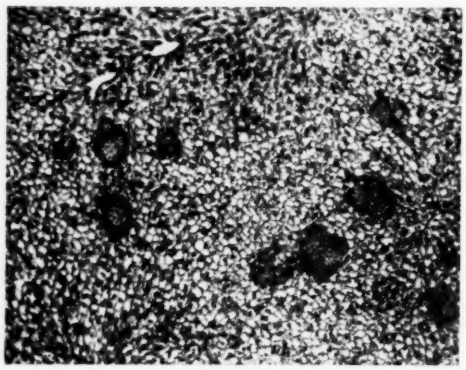

FIG. 3. LIVER FROM RABBIT PRETREATED WITH 1 MG. OF CORTISONE DAILY FOR FIVE DAYS AND THEN INFECTED WITH *Br. suis*. ANIMAL SACRIFICED ON FIFTH DAY OF INFECTION

Note the clear parenchymal cells with nuclei at the side. Several small granulomata with central necrosis. Hematoxylin and eosin, 60 ×.

cortisone-treated animals that had been infected with *Br. melitensis* and *Br. suis*. Organisms were noted in the hepatic cells of the rabbits infected with *Br. melitensis*.

A larger group of rabbits was then pretreated with cortisone, infected with *Br. suis*, and then treated for another two weeks. One cortisone-treated rabbit died on the eighth day of the infection, and two were critically ill at the time they were sacrificed on the fourteenth day. Just prior to death the Brucella skin test was negative in all the animals. Colonies of *Br. suis* were recovered from the bloods of all the animals that had survived the infection for two weeks (Table III, B). Agglutinins for Brucella were present in all the animals, and there was no difference in titer between the cortisone- and saline-treated groups. The weights of the spleens in the cortisone-treated animals were considerably reduced compared to the saline-treated controls. Colonies of *Br. suis* were cultured from the livers and spleens of all the cortisone-treated rabbits, and in greater numbers than were obtained from the saline-treated animals. The only significant lesions seen were in the livers. While the number of lesions was approximately the same in the cortisone and the saline-treated groups, the hepatic lesions in the cortisone-treated animals showed more necrosis and infiltration with polymorphonuclear leukocytes (Figures 4 and 5).

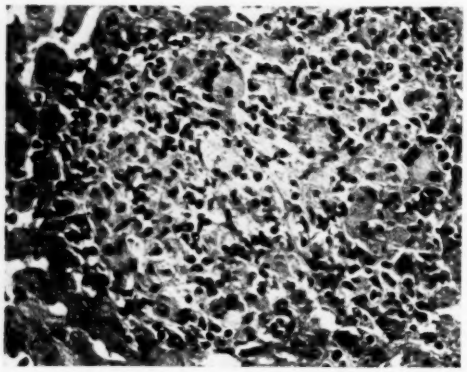

FIG. 4. LIVER FROM RABBIT PRETREATED WITH SALINE SOLUTION AND THEN INFECTED WITH *Br. suis*

Animal sacrificed after infection had endured for two weeks. Lesion shows an absence of necrosis and no polymorphonuclear leukocytes are included in the infiltration. Hematoxylin and eosin, 300 ×.

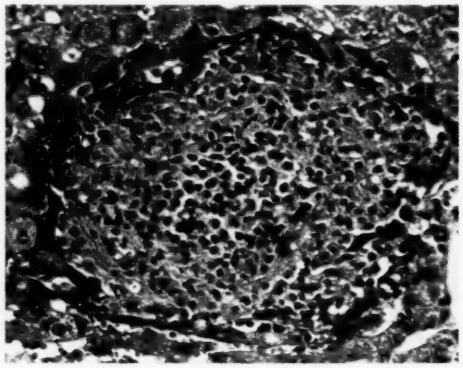

FIG. 5. HEPATIC LESION IN RABBIT PRETREATED WITH CORTISONE FOR FIVE DAYS AND THEN INFECTED WITH *Br. suis*

Animals sacrificed after infection had endured for two weeks. At the periphery of the lesion there are mononuclear cells. There is central necrosis with infiltration by polymorphonuclear leukocytes. Hematoxylin and eosin, 300 ×.

Influence of cortisone on chronic brucellosis

Mice. Mice were each infected with 100 million colonies of *Br. suis*, and then after a month were given 1 mg. of cortisone daily for a mean

TABLE IV

The influence of cortisone on chronic brucellosis in mice

A

After being infected with *Br. suis* one month previously, the animals were injected with 1 mg. of cortisone daily for an average of 11 days. Control infected animals were treated in similar manner with saline solution for 14 days.

Group	Number of animals	Average days of treatment	Average total days of infection	No. of animals with Brucella in heart's blood at time of death	Average wt. of spleens, grams	No. of animals with Brucella cultured from		No. of microscopic lesions per 10 low-power fields in			
						Liver	Spleen	Liver	Spleen	Kidney	Lung
Infected—Cortisone	12	11	41	0/0	0.101	1/12	0/12	10	0	0/12	0/12
Infected—Saline	12	14	44	1/11	0.176	2/12	4/12	7	0	2/12	1/11

B

Mice that had been infected with *Br. melitensis*, *Br. suis*, or *Br. abortus* 10 to 12 months previous to receiving 4 mg. of cortisone daily for average of 9 days, or 0.16 ml. of saline solution for average of 14 days. Two uninfected control mice received 4 mg. of cortisone daily for an average of 11 days.

Infected—Cortisone	11	9	10–12 mos.	0/1*	0.122	2/11	3/9	5/11	0	0/11	0
Infected—Saline	11	14	10–12 mos.	1/8*	0.304	2/11	0/9	4/11	0	3/11	0
Uninfected—Cortisone	2	11	—		0.040	0	0	0	0	0	0

* Blood from tail.

survival time of 11 days. Infected control mice were treated with saline solution. The data are presented in Table IV, A. The spleens of the cortisone-treated group were smaller than those receiving saline solution. But the outstanding feature was the lack of difference in the appearance of the hepatic lesions in the two groups. Only occasional granulomas were noted, and no bacteria were seen. The administration of relatively large doses of cortisone did not appear to alter the course of a chronic Brucella infection.

A heterogenous group of mice infected and kept in the laboratory for 10 to 12 months were treated as follows: nine mice were infected with *Br. melitensis*, five receiving 4 mg. of cortisone daily and three, 0.16 ml. of saline solution; five mice were infected with *Br. abortus*, two receiving cortisone and two, saline solution in the same doses as above; four mice were infected with *Br. abortus* following splenectomy, two receiving cortisone and two saline solution; four mice were infected with *Br. suis*, two receiving cortisone and two, saline solution. In addition to these 22 mice, two additional uninfected animals served as controls, each receiving 4 mg. of cortisone daily. The data are summarized in Table IV, B. The mean survival time of the cortisone-treated, infected animals was nine days, whereas that of the cortisone controls was 11 days. All the animals receiving saline solution survived the planned period of two weeks with therapy. An attempt was made to obtain blood cultures from each animal toward the end of the period of treatment by clipping the tail and allowing one or two drops of blood to fall on an agar plate. Only one positive blood culture was obtained—from a cortisone-treated animal from which *Br. abortus* was recovered. At the time the animals were sacrificed, an endeavor was made to obtain sufficient blood for the agglutination reaction and for blood cultures. No agglutinins were demonstrated in the bloods of two cortisone-treated animals, while four of nine mice that had received saline solution showed a mean titer of 1 to 320. The heart's blood of one cortisone-treated animal was cultured and yielded no Brucella organisms, whereas colonies of Brucella were recovered from one of the eight saline-treated mice. The mean weight of the spleens of the infected cortisone-treated group was considerably less than the saline-treated mice. Cultures of the organs for Brucella were positive in three of the cortisone-treated group; two of the animals having positive cultures from both the livers and spleens, and one from the spleen alone. In the saline-treated group, Brucella was isolated from only the livers of two of the animals. Various coliform organisms were recovered from the organs of the uninfected cortisone-treated controls. There was no difference in

TABLE V

The influence of cortisone on chronic brucellosis in guinea pigs

One guinea pig infected with *Br. melitensis*, one with *Br. suis*, and one with *Br. abortus*. After one month, animals were treated with 10 mg. of cortisone daily for 14 days. Three control animals, infected similarly, were treated with saline solution.

Group	Number of animals	Days of treatment	Days of infection	Brucella from heart's blood		Average wt. of spleens, grams	No. of animals with Brucella cultured from		No. of microscopic lesions per 10 low-power fields in			
				Before treatment	After treatment		Liver	Spleen	Liver	Spleen	Kidney	Lung
Infected—Cortisone	3	14	42	2/3	2/3	3.23	2/3	2/3	3	3	0	—
Infected—Saline	3	14	42	1/2	0/2	4.39	3/3	3/3	9	0	0	—

the appearance of the splenic and hepatic lesions in either the cortisone-treated animals or in the saline group. Very few hepatic lesions were found in any of the animals, regardless of the type of therapy. When lesions were present, they consisted of small granulomas composed of mononuclear and epithelioid cells, but without giant cells, necrosis or polymorphonuclear leukocytes. Each spleen from the infected animals contained giant cells and a reduced number of germinating follicles, which varied from animal to animal, but there was no correlation between the appearance of the lesions and the type of therapy.

Guinea pigs. Three pairs of guinea pigs were infected intraperitoneally with approximately one billion Brucella cells; one pair receiving *Br. suis*, one pair *Br. melitensis*, and one pair *Br. abortus*. One month later, one animal in each pair was treated with 10 mg. of cortisone daily for 14 days, and the three control animals were given saline solution. Just prior to starting therapy, and at the conclusion of five days of treatment, blood was obtained from each animal by cardiac puncture for cultures and for agglutination tests. Table V summarizes the results of this experiment. There was no essential difference in the results of blood cultures obtained before and after treatment with either cortisone or saline solution; nor was there any significant difference in the agglutinin titers. The skin reaction with Brucella antigen was less in the cortisone-treated animals. The weights of the spleens from the animals that had received cortisone were less than the saline-treated controls, but the differences were not so great as observed in the animals having more acute infections and treated with cortisone (see Table II, A and II, B). Brucella organisms were recovered from the livers and spleens in two out of the three cortisone-treated animals, and from the organs of all three saline-treated controls. There was no significant

TABLE VI

The influence of cortisone on chronic brucellosis in rabbits

A

One rabbit was infected with *Br. melitensis*, one with *Br. suis*, and one with *Br. abortus*. After one month, the animals were treated with 12.5 mg. of cortisone for 14 days. Three control animals infected similarly were treated with saline solution.

Group	Number of animals	Days of treatment	Days of infection	Brucella from heart's blood		Average wt. of spleens, grams	No. of animals with Brucella cultured from		No. of microscopic lesions per 10 low-power fields in			
				Before treatment	After treatment		Liver	Spleen	Liver	Spleen	Kidney	Lung
Infected—Cortisone	3	14	42	2/3	1/3	1.83	0/3	0/3	3	0	0	—
Infected—Saline	3	14	42	2/3	0/2	2.44	2/3	1/3	1	2	0	—

B

After being infected four months previously with *Br. abortus*, animals were injected with 12.5 mg. of cortisone daily for 14 days. One control animal similarly infected was treated with saline solution.

Infected—Cortisone	3	14	137	0/3	0/3	0.90	0/3	0/3	0	0	0	—
Infected—Saline	1	14	137	1/1	1/1	1.53	0/1	0/1	0	0	0	—

difference in the number of bacteria obtained from the organs of the two groups, nor in the microscopic examination of the livers, spleens, and kidneys. Only a few small lesions in the livers were seen.

Rabbit. Three pairs of rabbits were infected intravenously with about one and one-half billion Brucella organisms. One pair received *Br. melitensis*, one pair *Br. suis*, and one pair *Br. abortus*. One month later three animals were given 12.5 mg. of cortisone daily for 14 days, and three received saline solution. Skin tests performed just before the animals were sacrificed resulted in smaller nodules and less edema in the cortisone-treated rabbits than in the saline-treated controls. Blood obtained by cardiac puncture just before instituting treatment and at the time of sacrifice did not show any significant difference in the blood cultures and agglutination tests in the two groups of rabbits (see Table VI, A). The average weights of the spleens of the cortisone-treated animals were definitely less than the saline-treated controls. Cultures of the livers and spleens from the animals that had received cortisone did not reveal any Brucella, whereas these organisms were cultured from the livers of two of three saline-treated rabbits and from the spleen of one. A very few hepatic lesions were noted microscopically, but there was no difference in the appearance of the lesions in the cortisone-treated and saline-treated animals.

In another experiment with rabbits, animals were treated that had been infected for four months. Infection was established by the intravenous injection of *Br. abortus* in a total dose of 18.75 billion organisms given in five divided doses. Three of the animals were given 12.5 mg. of cortisone daily for 14 days, and one was given 0.5 ml. of saline solution for the same period. The results are presented in Table VI, B. Blood obtained by cardiac puncture from the animals before and after treatment with cortisone did not contain any Brucella, but *Br. abortus* was recovered both times from the single saline-treated control. The mean blood agglutinin titer of the cortisone-treated animals was 1 : 1,280 just before treatment, and 1 : 160 at the conclusion of treatment. The titer in the one saline control animal was 1 : 2,560 before treatment, falling to 1 : 640 after therapy. The mean weight of the spleens in the cortisone-treated animals was much less than that of the saline-treated control. No Brucella organisms were recovered from the livers or spleens of any of the animals, and no lesions were seen microscopically in the livers, spleens, and lungs.

DISCUSSION

The primary objective of the foregoing experiments was to measure the influence of cortisone upon the host-parasite relationship in experimentally infected animals having acute or chronic Brucella infections. Comparative observations were made in mice, guinea pigs and rabbits infected with *Br. abortus*, *Br. melitensis*, or *Br. suis*. In all three species of animals having an acute infection due to *Br. suis*, cortisone accelerated the multiplication of Brucella in the body and augmented the destruction of tissue, but this enhancement of bacterial dissemination was not so pronounced with *Br. abortus* and *Br. melitensis*. The most striking changes were demonstrated in mice having acute brucellosis. Cortisone converted a relatively mild infection into a rapidly fatal disease. The liver best demonstrated the tissue changes induced in these animals. Saline-treated control animals infected with *Br. suis* revealed a moderate number of granulomatous lesions in which little or no necrosis was apparent, and the cellular infiltrate included few or no polymorphonuclear leukocytes. This type of tissue reaction represents a good defense mechanism against invading Brucella. Cortisone abruptly and disastrously altered the host-parasite relationship in favor of the invading Brucella. The Brucella multiplied rapidly as evidenced by an intense parasitization of the hepatic parenchymal cells and an engorgement of the Kupfer cells by bacteria. This was associated with the destruction of the hepatic cells, with necrosis, and an infiltration by polymorphonuclear leukocytes. The deleterious effects of cortisone were accentuated by the larger doses and were most apparent in those animals which received cortisone for a few days before they were infected with Brucella. A similar enhancing effect has been reported by Tempereau and his associates (18) in guinea pigs having acute infections due to *Br. abortus* and treated with ACTH. It is of interest that in the present experiments cortisone did not appear to effect the course of the disease in guinea pigs, and it pro-

voked only a moderate enhancement of the infection in the rabbit.

In contrast to the effect of cortisone in acutely infected animals, the steroid failed to disturb significantly the host-parasite balance in the chronically infected animal. Protection of the animal with chronic disease against cortisone may have been afforded by an acquired immune mechanism against Brucella. This immunity could have prevented the multiplication of Brucella and caused the intracellular localization of the remaining organisms. While the administration of cortisone could result in the liberation of the intracellular organisms, an efficient humoral immunity would prevent the multiplication of the Brucella and invasion of other cells of the host.

Hypersensitivity, as measured by the skin reaction with Brucella antigen, was suppressed in the guinea pig and rabbit by cortisone. Suppression of skin reactions in other infections by cortisone and ACTH has been observed (19–21). Cortisone did not appear to cause a consistent change in the titer of Brucella agglutinins, nor did cortisone prevent the appearance of agglutinins in acutely infected animals. Neither ACTH nor cortisone interfere with the production of pneumococcic antibodies (22, 23).

One of the consistent side effects of cortisone was the diminution in the size of the spleen in the cortisone-treated animals as compared to the saline-treated group. In a previous study in mice by Braude and Spink (24) it was demonstrated that the spleen apparently protects the liver against Brucella. The hepatic lesions in splenectomized mice were more widespread than in infected control animals with spleens. In the present study, cortisone did not enhance the damage to the liver in splenectomized mice, as compared to the saline-treated animals.

SUMMARY

1. Cortisone was administered to mice, guinea pigs and rabbits having acute or chronic brucellosis due to *Br. abortus*, *Br. melitensis*, or *Br. suis*. Comparative histopathologic studies of the liver and spleen were made, as well as bacteriologic and immunologic observations.

2. The most striking effects of cortisone were manifested in mice that had acute brucellosis due to *Br. suis* and that were pretreated with the steroid. Under these circumstances, a relatively mild infection in which the liver revealed granulomatous lesions without necrosis was converted into a fulminating and fatal illness with a marked increase in the number of hepatic lesions. Quite impressive was the necrosis and destruction of parenchymal tissue, and Brucella organisms appeared to be multiplying in the hepatic cells and engorging the Kupfer cells.

3. In contrast to the abrupt changes induced in the acutely infected animals, cortisone did not appear to alter the course of the disease nor the tissue reactions in the chronically infected animals. It is suggested that an efficient immune mechanism protected the animals with chronic brucellosis against the enhancing effect of cortisone exhibited in the animals with acute infections.

4. Dermal hypersensitivity to Brucella antigen was suppressed by cortisone in guinea pigs and rabbits. Cortisone did not interfere with the appearance of Brucella agglutinins in the acutely infected animals. The titer of agglutinins in animals with chronic brucellosis was not consistently altered by cortisone.

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THE INFLUENCE OF CORTISONE AND ADRENOCORTICOTROPHIC HORMONE ON BRUCELLOSIS. II. ADRENOCORTICOTROPHIC HORMONE (ACTH) IN ACUTE AND CHRONIC HUMAN BRUCELLOSIS

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It has been established that Brucella organisms localize intracellularly within the tissues of animals and human beings (1–6). As pointed out elsewhere (7, 8), the majority of patients with brucellosis recover from their illness within a year. While there is no doubt that the duration of the illness in the majority of patients is considerably shortened by the administration of antibiotics, there still remains a small but significant number of patients who continue to have manifestations of active infection after what is considered to be adequate therapy (9). There is evidence that the chronicity of the disease may be due to the continued parasitization of the tissue cells by brucella organisms. Although effective therapeutic agents are available for brucellosis, *in vitro* investigations have demonstrated that Brucella organisms located in the cytoplasm of the cells of the host are protected against the action of the antibiotics, and also against the antibacterial action of serum (10, 11).

In tuberculosis, the invading bacilli tend to localize within the cells of the host, and possibly multiply there (12). Furthermore, it has been demonstrated in tissue cultures that the extracellular organisms of *Mycobacterium tuberculosis* are readily destroyed by streptomycin, but those bacilli within the macrophages are protected against the lethal action of the drug (13). Hart and Rees (14) have reported that tubercle bacilli are widely disseminated in mice with acute and chronic tuberculosis following the administration of cortisone. Apparently, the dissemination of tubercle bacilli from localized areas as a result of treatment with cortisone is not controlled by an extracellular immune mechanism, since the organisms multiply and invade other cells. Abernathy and Spink (15) observed that the administration of cortisone in mice having acute brucellosis results in the dissemination and multiplication of Brucella organisms, and a fulminating and fatal infection ensues. However, in the chronically infected animal, cortisone was without demonstrable effect, probably due in part to an efficient immune mechanism that prevented multiplication of the bacteria and invasion of other cells of the host.

Although experimental and clinical observations have demonstrated quite clearly that the continued illness of both animals and human subjects can be due to the persistence of Brucella organisms in the tissues, it has been difficult to demonstrate the intracellular localization of the Brucella. An analogous situation occurs in rickettsial diseases. It is an accepted fact that the rickettsiae invade the host's tissue cells, but demonstration of the intracellular position of the parasites in human material is often accomplished only after extensive search. Accepting the thesis that Brucella organisms localize and persist intracellularly, ACTH was administered to a group of patients having an acute or chronic illness due to Brucella in the anticipation that the organisms would be released from their intracellular location. It was further anticipated that the extracellular bacteria would be destroyed by bactericidins alone, or with the aid of antibiotic therapy.

Although the primary purpose in administering ACTH to chronically ill patients was to determine if Brucella organisms would be liberated from foci in the body, another objective was to observe the influence of the steroid on the symptomatology that

[1] Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

frequently persists in patients who have had an acute attack of brucellosis. These subjective manifestations consist of weakness, mental depression, nervousness, anorexia, and vague somatic aches and pains. As discussed elsewhere (7), one cannot always be certain whether these complaints represent the residuals of a previous infection or whether they indicate the presence of a continuing infectious process. There existed the possibility that, whatever the genesis of the symptomatology, the chronically ill patients might not only be temporarily benefited by ACTH, but a better state of health might ensue after its discontinuance.

METHODS OF STUDY

Seven male patients received ACTH.[2] Two of the seven patients had acute brucellosis, that is, an illness of less than three months' duration. The remaining five patients had a chronic illness with symptoms enduring for many months. ACTH was administered parenterally in doses of 20 to 25 mg. every six hours for seven to 14 days. Just prior to receiving ACTH, several procedures were carried out on the patients as a control on the influence of ACTH. Specimens of venous blood were obtained and added to trypticase-soy broth for cultural purposes. The agglutination test was performed with the serum utilizing the tube-dilution technique and a *Brucella abortus* antigen supplied by the Bureau of Animal In-

[2] Supplied by Armour Laboratories.

TABLE I

Pertinent data of patients with brucellosis given ACTH

No. of pt.	Age and sex	Occupation	Duration of symptoms before receiving ACTH	Laboratory data: Before ACTH — Reciprocal of Brucella titer	Laboratory data: Before ACTH — Blood culture	Laboratory data: During and after ACTH — Reciprocal of Brucella titer	Laboratory data: During and after ACTH — Blood culture	ACTH dose	Comment
1	30 Male	Laborer, meat packing plant	6 weeks	[640]	*Br. abortus*	640	0, 0, 0, 0 0, 0, 0	20 mg. 4 times daily for 10 days	Improvement; no relapse; good health 12 months later.
2	34 Male	Farmer	8 weeks	[1,280]	[*Br. abortus* *Br. abortus* *Br. abortus*]	1,280 1,280	*Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus* *Br. abortus*	25 mg. 4 times daily for 11 days	Immediate and marked improvement, although blood cultures remained positive. Subsequent treatment with combined aureomycin and dihydrostreptomycin. Recovery, and good health for 3 months.
3	28 Male	Farmer	9 months	1,280 1,280 320 160 [±160]	*Br. abortus* *Br. abortus* 0 [0, 0, 0, 0]	±160	0, 0, 0, 0	20 mg. 4 times daily for one week	Improvement; increase in strength; no relapse; working 12 months later. Mild complaints.
4	54 Male	Farmer	3 months	[640]	[0, 0, 0]	640	0, 0, 0, 0 0, 0, 0, 0 0, 0, 0, 0 0, 0	25 mg. 4 times daily for 10 days	Improvement; relapse of symptoms in 2 mos. Had positive serology for syphilis and fracture of right ankle.
5	56 Male	Farmer	27 months	640 2,560 [640]	*Br. abortus* *Br. abortus* [0, 0, 0, 0, 0, 0]	160 320	0, 0, 0, 0 0, 0, 0,	25 mg. 4 times daily for 14 days	Improvement; relapse in few days. Given aureomycin. Working 12 months later—still has aches and pains.
6	35 Male	Electrician, meat packing plant	16 months	320 640 320 640 320 [640]	*Br. abortus* *Br. abortus* [0, 0]	1,280 1,280	0, 0, 0, 0 0, 0, 0	20 mg. 4 times daily for 10 days	Marked improvement; no relapse; good health for 9 mos., then entered hospital for spontaneous pneumothorax. Recovery.
7	51 Male	Farmer	9 months	[160]	[0, 0, 0]	160	0	32.5 mg. 4 times daily for 14 days	Patient had destruction of glenoid process of right shoulder resulting in shoulder-hand syndrome and disability. Simultaneous with ACTH received 0.5 gm. aureomycin 4 times daily for 3 weeks along with 0.5 gm. dihydrostreptomycin twice daily for 2 weeks. Marked improvement which has continued for 3 mos. since treatment. Now back at work.

[ ] = Laboratory data obtained just prior to administration of ACTH.

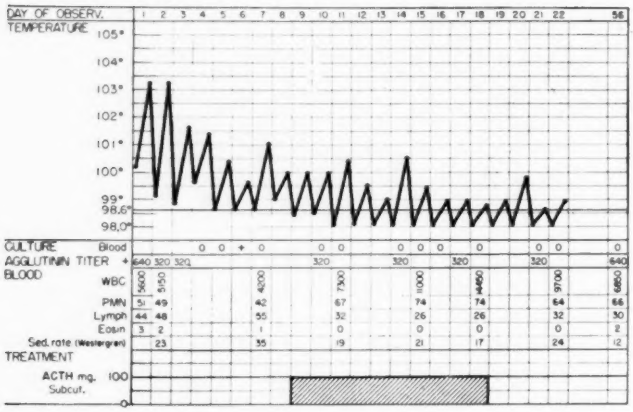

FIG. 1. A. C., 30-YEAR-OLD WHITE EMPLOYEE OF A MEAT PACKING PLANT, TREATED WITH ACTH

Note positive blood culture due to *Br. abortus* before receiving ACTH and sterile cultures during and after administration of ACTH. No relapse within follow-up period of one year.

dustry. Incubation in a water bath at 37° C. was carried out for 24 to 48 hours. Total leukocyte and differential counts were made. These same procedures were repeated during and after the course of treatment with ACTH. While the patients were receiving ACTH, the influence of the hormone on dermal hypersensitivity to Brucella antigen was determined. The patients were then checked periodically for a minimum of three months, and up to a year, before this report was prepared.

RESULTS

Pertinent data relating to each patient are presented in Table I with a comprehensive and detailed history in order to eliminate any doubt about the nature of the illness, and to portray the difficult problem of contending with the more chronically ill patients.

ACTH in acute brucellosis

Case 1. A. C., a 30-year-old white man was admitted to the hospital because of chills, fever, sweats, and weakness of one month's duration. Approximately six months prior to the onset of his illness he had terminated his employment in a meat packing plant, where he was daily exposed to the tissues of cattle condemned for slaughter because of Bang's disease. He had been treated successfully for a brief period with aureomycin. He had a temperature of 103° F. and a pulse rate of 118 per minute. The only abnormalities noted were slightly enlarged cervical lymph nodes, and bilateral tenderness of the testicles. He had a total leukocyte count of 5,600 cells per cu. mm. with 51 per cent neutrophiles and 44 per cent lymphocytes. The erythrocyte sedimentation rate (Westergren) was 23 mm. in one hour. Brucella agglutinins were present in a titer of 1 to 640. One of three blood cultures yielded *Br. abortus.*

After a brief period of observation at bed rest the patient was given 25 mg. of ACTH subcutaneously four times daily for 10 days. A graphic presentation of his clinical course is shown in Figure 1. While he was receiving ACTH there was no abrupt subjective or objective change in the condition of the patient. The temperature declined by lysis. Five specimens of venous blood taken during the period of ACTH administration and two obtained after the injections were discontinued remained sterile. ACTH did not induce any significant changes in the agglutinin titer. One interesting laboratory finding was an increase in the leukocyte count to 14,450 cells during ACTH therapy, with 76 per cent neutrophiles and 26 per cent lymphocytes. This is an unusual observation during the course of acute brucellosis. The erythrocyte sedimentation remained essentially unchanged. During thirty days after the conclusion of the injections of ACTH, he remained well and afebrile. There were no abnormal physical findings. The titer of Brucella agglutinins was 1 to 640, and three specimens of venous blood obtained on three consecutive days remained sterile. In the year following the administration of ACTH he had not had a relapse.

Comment: Contrary to expectations, a febrile patient with acute brucellosis and a bacteremia due to *Br. abortus* recovered from his illness following the administration of ACTH. The only apparent significant hematologic influence of ACTH was

an increase in the total leukocyte count and a diminution in the number of lymphocytes and eosinophile cells.

Case 2. W. J. K., a 34-year-old white tenant farmer had the onset of an illness eight weeks prior to entering the hospital. The initial manifestations were chills and a fever of 104° F. He took aureomycin for a brief period of time, and he became afebrile; but he continued to feel weak and he had a relapse of chills and fever. He then received aureomycin and penicillin, and concomitantly his temperature became normal, but after several days there was a recurrence of daily chills and fever. Until he presented himself for his final hospital admission in Minneapolis the diagnosis of brucellosis had not been established. He worked with a large herd of high-grade dairy cattle, and although there had not been any abortions in the herd for three years, he had assisted the local veterinarian in vaccinating the calves twice yearly with a viable culture of strain 19 *Br. abortus*. The herd had not been tested recently for Bang's disease.

He appeared quite toxic with a temperature of 101.4° F. There were no abnormal physical findings other than the edge of a tender liver 4 cms. below the costal margin. Jaundice was not present. Prior to the administration of ACTH the total leukocyte count was 5,000 per cu. mm. with 52 per cent neutrophiles and 46 per cent lymphocytes. The erythrocyte sedimentation rate of 15 mm. in one hour was within normal limits. Brucella agglutinins were present in a titer of 1 to 1,280. *Br. abortus* was cultured from four specimens of venous blood obtained on consecutive days. Liver function studies showed 10 per cent bromsulfalein retention, normal serum bilirubin, normal prothrombin time, 2+ cephalin flocculation, thymol turbidity of 7.6 units and zinc sulfate turbidity of 12.9 units. Stained sections of sternal bone marrow aspirated on the day treatment with ACTH was begun revealed multiple granulomas. A section of liver acquired on the same day showed localized areas of lymphocytes, epithelioid cells, and fibroblasts characteristic of granulomatous inflammation.

After the patient had been observed in bed for a few days, ACTH was administered in a dose of 25 mg. intramuscularly every six hours for 11 days. The course of events is presented graphically in Figure 2. Within 15 hours after the first injection of ACTH his temperature returned to normal and was never above 100° F. during hormone treatment. By the fourth day he felt much improved. At this time the liver was notably smaller and the tenderness had abated. He was ambulatory on the seventh day. The following day a skin test with a carbohydrate fraction of Brucella induced a very slight reaction. The leukocyte count rose from 3,600 to 7,600 with 82 per cent neutrophiles. The erythrocyte sedimentation rate remained within normal limits. The titer of Brucella agglutinins was the same. But cultures of the blood continued to show the presence of *Br. abortus* (Figure 2). Liver function tests remained essentially unchanged. Sections of the liver obtained at the conclusion of 10 days' ACTH administration showed approxi-

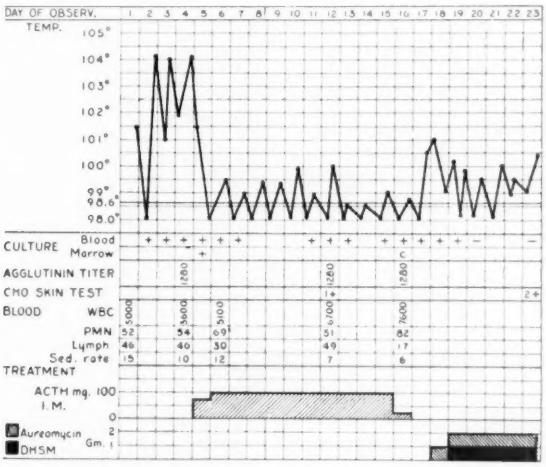

FIG. 2. CASE 3. W. J. K., A 34-YEAR-OLD FARMER TREATED WITH ACTH

Note the drop in temperature coincident with administration of ACTH, and persistence of bacteremia due to *Br. abortus*. Treated subsequently and successfully with aureomycin and dihydrostreptomycin.

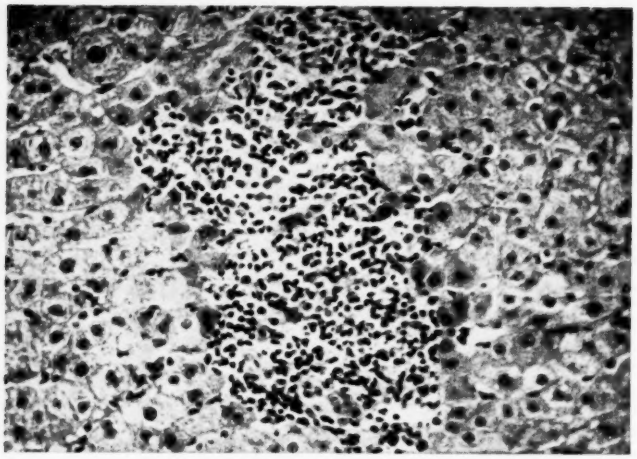

FIG. 3. SPECIMEN OF LIVER FROM CASE 2 SHOWING A GRANULOMA OBTAINED BEFORE THE ADMINISTRATION OF ACTH

Hematoxylin and eosin. 350 ×.

mately the same type and extent of cellular reaction as did those just prior to the beginning of treatment (Figures 3 and 4). Likewise, sections of sternal bone marrow revealed the same appearance of granulomas before and after treatment. On the day that ACTH was discontinued the patient had no complaints. No physical abnormalities could be demonstrated. Because of the persistent bacteremia and a slight elevation of temperature, simultaneous treatment with aureomycin and dihydrostreptomycin was instituted. He received 0.5 gm. of aureomycin orally every six hours for three weeks. Dihydrostreptomycin was administered intramuscularly in a dose of 0.5 gm. every 12 hours for two weeks. Shortly after this combined treatment was started the bacteremia was eradicated. He continued to feel well and has had no further relapses three months after the conclusion of treatment.

Comment: The most striking feature was the abrupt improvement that occurred within 24 hours after the first injection of ACTH. A toxic and listless patient became alert and afebrile. The remarkable aspect is that such a state of well-being persisted although Brucella organisms still could be cultured from the blood. Another interesting observation was the decrease in the size and tenderness of the liver induced by ACTH, although there was no essential change in the inflammatory reaction. As in Case 1, ACTH influenced the leukocytes in the peripheral blood with a shift from a relative lymphocytosis to an increase in the polymorphonuclear cells and an elevation in the total leukocyte count.

ACTH in chronic brucellosis

Case 3. G. M., a 28-year-old white farmer had had an acute illness of two weeks' duration with chills, sweats, nausea and vomiting. Because of a duodenal ulcer he had ingested large amounts of unpasteurized milk. He did

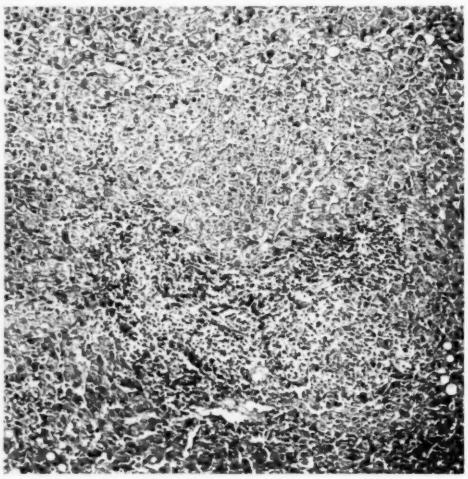

FIG. 4. SPECIMEN OF LIVER OBTAINED FROM CASE 2 AFTER ACTH HAD BEEN ADMINISTERED FOR 11 DAYS

A granuloma is demonstrated. There was no essential change in the histology of the liver. Hematoxylin and eosin. 100 ×.

not know of any Bang's disease in his herd of cattle, but his neighbor's cattle in an adjoining pasture had the disease. His temperature was 104° F., and he had lymphadenopathy, hepatomegaly, and an enlarged and tender spleen. The titer of Brucella agglutinins was 1 to 1,280, and *Br. abortus* organisms were isolated from the blood on two occasions. He was given aureomycin, and then terramycin, after which his symptoms subsided. The spleen was still palpable at the costal margin. Three cultures of blood taken on consecutive days remained sterile, and the titer of agglutinins was 1 to 1,280. A month later he was still without symptoms and afebrile, but the spleen was just palpable. Brucella agglutinins were present in a titer of 1 to 320, and a blood culture remained sterile. Six months later he complained of weakness. He was afebrile, and the spleen could not be felt. Two blood cultures remained sterile, and the titer of agglutinins was 1 to 160.

He was readmitted to the hospital nine months after his initial hospitalization because he tired easily. He admitted using alcohol excessively. He was afebrile. The spleen was not palpable. The total leukocyte count was 7,000 with 72 per cent neutrophiles and 19 per cent lymphocytes. The erythrocyte sedimentation rate was normal. An agglutination test showed incomplete clumping of the brucella antigen up to a titer of 1 to 160. Two other agglutination tests revealed no agglutinins in his serum. Daily cultures of blood for three days remained sterile. He was given 20 mg. of ACTH subcutaneously four times daily for one week. During this time the patient felt stronger, but there were no objective changes noted. There were no alterations of the leukocyte counts or of the sedimentation rate. On the sixth day of therapy with ACTH there was no immediate skin reaction following the intradermal injection of 0.1 ml. of a 1 to 100 dilution of Brucella carbohydrate, and 24 hours later only a moderate reaction was seen. A skin test with heat-killed Brucella cells also showed a moderate reaction of the delayed type. After 15 days of hospitalization he was discharged. He felt well. When examined three months later he still felt well. At this time the antigen was only partially agglutinated by his serum in a titer of 1 to 160, and two blood cultures remained sterile. He has continued in good health for a year.

Comment: This was the first patient with presumed chronic brucellosis who was treated with ACTH. Although there is no doubt that he had acute brucellosis, there is some question as to whether a chronic infection was responsible for his ill health at a later time. He felt weak, but he was an alcoholic. He had a declining titer of Brucella agglutinins, and the test showed incomplete clumping of the antigen, which is so characteristic of the serum of those patients who have recovered from brucellosis. It is of interest that ACTH diminished but did not abolish the delayed type of dermal hypersensitivity to Brucella antigens.

Case 4. A. H., a 54-year-old white farm worker who had chills, fever, sweats, headaches, weakness, and epigastric distress about three months before entry into the hospital. Shortly before, he had fractured his ankle. At the time he became ill he worked on a farm where he drank unpasteurized milk and a cow had aborted. His temperature was normal. The only observed abnormality was a swollen and stiffened right ankle. The total leukocyte count was 8,600 with 56 per cent neutrophiles and 42 per cent lymphocytes. The erythrocyte sedimentation rate was 26 mm. in one hour and Brucella agglutinins were present in a titer of 1 to 640. Three cultures of blood taken on consecutive days remained sterile.

He was given 25 mg. of ACTH subcutaneously four times daily for ten days. Coincident with this he remained afebrile, felt well and gained nine pounds. There was no pitting edema, but after the injections of ACTH were discontinued, a diuresis occurred and he lost all the gain in weight. There was no significant change in the titer of agglutinins. Ten cultures of blood taken during and after ACTH treatment remained sterile. His leukocyte count rose to 11,500 with 84 per cent neutrophiles and 15 per cent lymphocytes. The sedimentation rate declined to 8 mm. in an hour. He felt well when he left the hospital 18 days after entry. About six weeks later he returned complaining of weakness and epigastric distress. He was afebrile and there was evidence of a poorly uniting fracture of the ankle. The titer of Brucella agglutinins was 1 to 640. Five cultures of blood remained sterile. Serologic tests for syphilis were positive and he admitted having had a primary infection 25 years previously treated with two long courses of injections. The cerebrospinal fluid was normal. A few days after this second appearance at the hospital he had evidence of a pulmonary infarction with a slight pleural effusion. Following rest in bed and treatment with aureomycin and penicillin he left the hospital feeling well. He was fitted with a brace to aid in the healing of the fracture. He has had no further relapse.

Comment: It is very likely that this patient had had an attack of acute brucellosis, and the evidence favored a continuation of the disease when he was seen at the hospital three months after the onset. The ACTH did not appear to alter his clinical course significantly.

Case 5. L. J. L., a 56-year-old white farmer had been ill for two years before his first appearance with chills, fever, sweats, dizziness, weakness and headaches. He drank unpasteurized milk and one of his cows had aborted. He appeared chronically ill with a temperature of 100.2° F. There were no demonstrable physical abnormalities. The total leukocyte count was 3,700 with 60 per cent neutrophiles and 32 per cent lymphocytes. The erythrocyte sedimentation rate was 57 mm. in one hour. The

titer of Brucella agglutinins was 1 to 640, and *Br. abortus* organisms were isolated from two cultures of blood. He was given 0.5 gm. of aureomycin orally every six hours for 21 days. He became afebrile and symptomatic, and the titer of Brucella agglutinins was 1 to 2,560.

He returned three months later feeling better than on his previous entry, but he still had a low-grade fever, cough, diarrhea, and became fatigued readily following physical exertion. He was afebrile and there were no unusual physical abnormalities. The leukocyte count was 5,500 with 64 per cent neutrophiles and 28 per cent lymphocytes. The erythrocyte sedimentation rate was 40 mm. in one hour and the titer of Brucella agglutinins 1 to 640. Six cultures of blood remained sterile. He was then given 25 mg. of ACTH subcutaneously four times daily for 14 days. During this time he developed a mild euphoria. His temperature remained normal. Prior to receiving ACTH he had a mild tremor of his hands, which felt cold and clammy. These manifestations diminished during the course of ACTH. There was no change in the titer of agglutinins, and nine cultures of blood observed during and after the administration of ACTH remained sterile. The patient stated that shortly after the injections of ACTH had been discontinued, and while he was at home, he developed severe weakness and marked tremor of the hands. His temperature rose to 99.6° F. A month later he returned. There were no abnormal physical findings. The titer of Brucella agglutinins was 1 to 160. Two cultures of blood remained sterile. He was then treated simultaneously for 12 days with 0.5 gm. of aureomycin every six hours and 0.5 gm. dihydrostreptomycin given intramuscularly twice daily. Treatment was discontinued because of the appearance of weakness, anorexia, and a display of hostility. An elevation of eosinophile cells to 12 per cent occurred. His behavior was thought to be an unusual type of reaction to the treatment, because as soon as the drugs were withheld the symptoms abated and the eosinophilia disappeared. His Brucella agglutinins showed a titer of 1 to 320, and nine cultures of blood remained sterile during and after treatment. He remained afebrile up to three months later. The titer of agglutinins was 1 to 80, and a blood culture was sterile.

Comment: This represented an unusual case of chronic brucellosis in our experience, since the patient had been ill for two years without any localizing manifestations and at the end of this time *Br. abortus* was cultured from his blood. He appeared to improve following treatment with aureomycin. His blood cultures remained sterile, although many specimens were studied. It was concluded that he still had active disease just prior to his receiving ACTH. Temporary improvement occurred while ACTH was being administered but the manifestations of an active disease appeared shortly thereafter. He apparently responded satisfactorily to treatment with aureomycin and dihydrostreptomycin.

Case 6. A. J., a 35-year-old white electrician employed in a meat packing plant first became ill with joint pains, nocturnal sweats, chills, cold feet, and fatigue. After two months of continued illness he was sent to a hospital for pulmonary tuberculosis because an X-ray film of the chest revealed a diffuse infiltration. Extensive studies eliminated tuberculosis. Although only a low-grade fever was present, *Br. abortus* was isolated from blood cultures on two occasions. He was given 2 gm. of chloramphenicol daily for 10 days and felt better. It was concluded that the pulmonary changes were unrelated to his brucellosis. A film of the chest taken some time before the onset of his illness had revealed lesions in the lungs. It was brought out on questioning that as an electrician he had broken fluorescent light tubes on many occasions, and berrylium intoxication was considered but never proven as the cause of the findings in the lung. He continued well until seven months after his discharge from the hospital when he suffered from pain in the chest, sweats, nervousness, backache and fatigue. Although blood cultures remained sterile, Brucella agglutinins were present in a titer of 1 to 640. He was given 0.5 gm. of aureomycin four times daily for two weeks after which he felt improved.

Four months later, or 16 months after the onset of his illness, he presented himself again because of nervousness, weakness, and sweats. It was decided to administer ACTH. He was afebrile and no abnormal physical findings could be detected. The leukocyte count was 6,850 with 81 per cent neutrophiles and 16 per cent lymphocytes. The sedimentation rate was normal. The titer of brucella agglutinins was 1 to 640 and a culture of blood remained sterile. He was given 20 mg. of ACTH subcutaneously every six hours for 10 days. A remarkable change took place in this patient within 24 hours. His mental depression subsided and his appetite increased considerably. The patient stated that he felt better than he had for many months. On the fifth day an intradermal test with brucellergen revealed a very slight reaction in 24 hours. The agglutinin titer was 1 to 1,280, and seven cultures of blood during and after the administration of ACTH remained sterile. Fifteen hours after the injections of ACTH had been discontinued the patient experienced excrutiating pains in both knees with radiation to a point between the knees and ankles. Morphine was necessary to allay the pain. There was no redness or swelling of the extremities. Within four days the pain had completely subsided. The patient went back to work and was well until nine months later when he had a spontaneous pneumothorax. After a brief period of hospitalization the lung re-expanded. At this time his Brucella agglutinins were 1 to 640, and blood cultures were sterile. He returned to work.

Comment: Of all the patients with chronic brucellosis who received ACTH this patient experi-

enced the greatest benefit. His illness was complicated by a diffuse pulmonary infiltration of doubtful etiology, which was probably unrelated to his brucellosis, and not benefitted by ACTH.

Case 7. H. J., a 51-year-old white farmer nine months prior to being studied had had a sore throat, pain in both shoulders, and bilateral orchitis. He had been treated with an antibiotic which was followed by partial relief of his symptoms. Two months later he had a "kidney infection" and then he developed a severe pain in his right shoulder. Subsequently the pain extended down the arm and involved the right hand so that his fingers became stiffened and movement was painful. He was incapacitated from working as a farmer. He had a herd of 31 cattle in which abortions had occurred, but no tests for Bang's disease had been made. He was afebrile. There was muscular atrophy in the region of the right shoulder, and pressure over the acromial area elicited pain. The fingers of the right hand were swollen and flexion was impaired. The right arm could not be elevated above the level of the shoulder. The white blood count was 7,700 with 64 per cent neutrophiles and 24 per cent lymphocytes. The sedimentation rate was normal. The brucella agglutinin titer was 1 to 160, and blood cultures remained sterile. An X-ray film of the right shoulder revealed marked decalcification of the right humerus, especially the head. In addition, there was decalcification of the glenoid process and evidence of destruction along one margin of the rim of the glenoid.

He was treated simultaneously with dihydrostreptomycin, aureomycin and ACTH. He was given 0.5 gm. of dihydrostreptomycin intramuscularly twice daily and 0.5 gm. of aureomycin orally every six hours for two weeks. At the same time he received 32.5 mg. of ACTH intramuscularly every six hours for two weeks. Therapy with aureomycin alone was continued another week. Physiotherapeutic measures for the right arm were also instituted. Under this regimen there was gradual improvement. There was no change in his agglutinin titer and a blood culture remained sterile. When seen two months after the completion of his treatment, he was back at work. There had been continued improvement, but there still was some limitation in movement of the right arm.

Comment: This patient was considered to have had a severe attack of acute brucellosis nine months prior to receiving treatment with ACTH. The severity of his illness was evidenced by the presence of bilateral orchitis and a destructive lesion of the joint of the right shoulder. He had received antibiotic therapy in amounts sufficient to control the infection partially, but not enough to prevent the appearance of an unusual manifestation of brucellosis, that of a shoulder-hand syndrome. Because of the destructive lesion of a joint it was deemed inadvisable to administer ACTH without affording the patient protection with an antibacterial agent or agents. ACTH appeared to be desirable therapeutically in view of the stiffened fingers of the right hand. For these reasons, antibacterial drugs and ACTH were administered simultaneously, and coincident with this therapeutic approach there was definite improvement in the condition of the patient. His health was restored enough to permit him to resume work.

DISCUSSION

The most striking effects of ACTH in human brucellosis were elicited in those patients who had had symptoms for less than three months (Cases 1 and 2). Both of these patients had a demonstrable bacteremia due to *Br. abortus* just prior to the administration of ACTH. The hormone did not have any deleterious effect on the clinical course of either patient. While no abrupt change was noted in Case 1, the patient did improve and there was no further evidence of bacteremia after the injections of ACTH were instituted. This patient did not suffer from any ill effects after the injections were discontinued, and he has remained well for 12 months. The sequence of events occurring in Case 2 as a result of ACTH may shed further light on the basic mechanisms of disease in brucellosis. In the first place, a febrile patient having a very toxic appearance was converted into an afebrile individual having the appearance and feeling of decided improvement within 24 hours after the initial injection of ACTH. Although therapy with the antibiotics will provoke such improvement, the changes have not been so abrupt nor have they occurred so quickly (9). This immediate improvement manifested itself in Case 2 although *Br. abortus* could readily be cultured from the blood. Similar responses have been observed in patients with pneumococcic infections and those with typhoid fever treated with ACTH or cortisone (16–18). Besides the clinical improvement displayed by Case 2, there were other objective changes that occurred in both Cases 1 and 2. A very common laboratory finding in patients with active brucellosis is a normal leukocyte count, or a leukopenia, with a relative lymphocytosis. During the administration of ACTH in Case 1 the leukocyte count rose to 14,450, a level which is

rarely seen in brucellosis and there was an increase in the polymorphonuclear cells with a relative decline of the lymphocytes. Similarly, in Case 2 there was rise in the leukocyte count, an increase in the number of neutrophile cells and a relative depression of the lymphocytes. ACTH did not appear to alter the titer of Brucella agglutinins, but dermal sensitivity to Brucella antigen was suppressed in Case 2.

Another provocative series of observations made in Case 2 related to the tissue reactions induced by Brucella and the influence of ACTH upon these reactions. It is to be recalled that in Case 2, prior to the administration of ACTH, the liver was enlarged and tender, and the results of liver function studies were abnormal. In addition, a specimen of liver showed the presence of hepatitis, which is a common finding in human brucellosis (19). Likewise, aspirated sternal bone marrow showed the characteristic granulomatous type of inflammation (20). It was anticipated that the administration of ACTH might intensify these inflammatory reactions, but on the contrary, the tenderness and enlargement of the liver decreased shortly after the injections were begun, and specimens of liver and bone marrow examined after the completion of the injections of ACTH did not reveal any essential change. During the course of ACTH administration there were no alterations in the functional studies of the liver as compared to the studies carried out just before the start of ACTH administration.

The abrupt clinical improvement manifested by Case 2 without eradication of the bacteremia and without any change in the inflammatory reaction of the tissue suggested that ACTH inhibited the toxic action of a product or products (? endotoxin) of Brucella upon the body. It has been observed in this laboratory that the simultaneous administration of cortisone will protect mice against the lethal effect of Brucella endotoxin (21).

The observations on experimental brucellosis in animals, especially in mice, which preceded these in human brucellosis, revealed that if animals were pretreated with cortisone and then infected with Brucella organisms, a fulminating and lethal infection ensued (15). Specimens of the liver showed necrosis of the hepatic cells and tremendous numbers of Brucella organisms invading the parenchymal cells and engorging the macrophages in the liver sinusoids. In the control animals that had not received cortisone, the infection was relatively mild, the inflammatory reaction was of the granulomatous type with little or no necrosis, and proliferation of the bacterial cells not marked. No such a sequence of events was observed in the human cases of acute brucellosis treated with ACTH. Perhaps the essential difference is not that the animals received cortisone and the humans ACTH, but rather that cortisone was offered to the infected animals before any immune response to the infection had been established, whereas the human patients had been ill for a period of time sufficient to permit the development of an effective antibacterial mechanism. While much remains to be learned about tissue and humoral immunity in brucellosis, the observations in the chronically infected animals and human beings indicate that the immune mechanism is efficient enough to prevent a dissemination of the infection when cortisone or ACTH is administered. This is contrary to observations made in experimental and human tuberculosis (14, 22, 23).

The administration of ACTH to patients having chronic brucellosis appeared to have little effect on the course of the illness except in one or two instances. Patient 7 was in a depressed mental state prior to receiving the hormone, but during its administration he experienced a feeling of well-being that endured for several months after the injections had been discontinued. ACTH did not have any undesirable effects on any of the five patients with chronic brucellosis. The hormone did suppress the dermal hypersensitivity to Brucella antigen, but it did not significantly alter the titer of Brucella agglutinins. In no instance, did Brucella organisms appear in cultures of blood during and after the administration of ACTH. It was anticipated that the administration of ACTH to patients with chronic brucellosis might liberate Brucella organisms from their foci and a bacteremia might be demonstrated. Failure to elicit this result might be related to the relatively small number of bacteria released, and to a humoral immunity effectively destroying the organisms as they appeared in the blood.

Upon the basis of the present observations, and because therapy with the antibiotics is so effective in the majority of patients, there is little or no indication for using either cortisone or ACTH in the

treatment of human brucellosis. A further contraindication is the undesirable side effects induced by these agents. The one possible indication for the use of either cortisone or ACTH would be in a seriously ill and toxic patient. Even in such a case, treatment probably should be carried out only for a day or two, and simultaneous treatment should be started with streptomycin or dihydrostreptomycin, in combination with aureomycin or terramycin. A similar approach has been made in the treatment of typhoid fever (16, 17).

The present results with ACTH in acute brucellosis have stimulated a further consideration of the mechanism whereby patients appear to benefit following the intravenous injection of Brucella antigens. Such authorities as Janbon (24) in Montpellier, France; Signorelli (25) in Farrar, Italy; and Huddleson (26) in this country have called attention to the beneficial results that have occurred in patients with acute and chronic brucellosis following the intravenous injection of Brucella antigens. It is of interest that improvement in the patient's condition is usually associated with a febrile reaction and, at times, a shock-like picture with a drop in blood pressure. Although it is often asserted that the Brucella preparations are specific in their beneficial action, some doubt has been cast upon this conclusion by the observations of Löffler and his associates (27), who obtained equally good results following the injection of suspensions of colloidal silver. It might be postulated that the febrile reaction induced by these agents provoked a stress reaction which in turn stimulated the adrenals to produce corticosteroids with the results as presented in Cases 1 and 2. Another possibility is that the febrile reaction with its mild shock-like manifestations might be representative of a generalized Shwartzman reaction in which Brucella organisms are liberated from localized foci, and disposed of by an extracellular immune mechanism. Further studies along these lines are in progress at the present time.

SUMMARY

1. Adrenocorticotrophic hormone (ACTH) was administered to two patients with acute brucellosis and to five patients with chronic disease. The administration of ACTH to one of the patients with acute brucellosis, who was quite ill, was accompanied by a prompt and remarkable improvement although the blood cultures remained positive for *Br. abortus*. ACTH did not appear to have any significant effect on the clinical course of chronic brucellosis.

2. Specimens of liver and sternal bone marrow from one patient with acute brucellosis displaying granulomas showed no essential change in the type or degree of inflammation before and after the administration of ACTH.

3. ACTH had no significant effect upon the titer of Brucella agglutinins, but it did suppress dermal hypersensitivity to Brucella antigens. The tendency of Brucella organisms to cause a leukopenia and relative lymphocytosis was abolished by ACTH.

4. The only therapeutic indication for cortisone or ACTH at the present time in acute or chronic brucellosis is in patients who are quite ill and toxic. And even then, treatment should be carried out for only a few days and simultaneously with antibiotic therapy.

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LIGHT SCATTERING STUDIES OF FIBRIN CLOTS[1]

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INTRODUCTION

Hemostasis depends largely on the conversion of fibrinogen to fibrin, catalyzed by the enzyme thrombin. In recent years, there has been considerable advance of knowledge of the formation of thrombin from its various precursors. However, a comparatively small amount of effort has been directed toward studies of the physical nature of the fibrin clot as a possible guide in the better understanding of hemostatic mechanisms in health and disease. In 1947 Ferry and Morrison (1, 2) converted purified human fibrinogen to fibrin under various conditions of pH, ionic strength, and fibrinogen concentration, and they measured such properties of the clot as opacity, rigidity, friability, and so forth and proposed two general structures: "The fine and the coarse clot." The fine clot showed small deviation from Rayleigh's law which states that the opacity is inversely proportional to the fourth power of the wavelength, but the coarse clot showed considerable departure from this proportion. They stated: "In a solid structure, such as fibrin clot, it is impossible to calculate explicitly the size and shape of structural units from opacity measurements." The larger strands have been studied by direct microscopic observation (3, 4) but the average size strand is too narrow for the limits of optical resolution. The diameter of the fibrin strands has also been measured by the electron microscope (5–8). This instrument, however, has the inherent objection of drying the clot with collapse of the three dimensional network. In the studies to be reported herein, light scattering as a method of determining fibrin strand size has been attempted.

THEORY[3]

Although the fundamental theory of the molecular scattering of light was given by Lord Rayleigh in 1871, only within the past decade has this method been of practical value in the measurement of the size and shape of particles of biological interest, for P. Debye emphasized that if the particles are comparable in size to the wavelength of light and have a relative index of refraction[4] near unity, then the system might be treated similarly to a problem in x-ray diffraction. There are several reviews of this subject (9–13). Light scattering techniques have been used to determine the size and shape of the fibrinogen molecule and of polymerization of the fibrinogen before gelation (14). However, there are no reports of a theory or techniques for dealing with particles which have the size and shape of fibrin strands. Therefore, a generalized theory of light scattering for cylindrical rods with a relative index of refraction near unity has been developed. The normalized expression for the angular scattering of light by randomly oriented cylindrical rods of length L and radius R is given by:

EQUATION 1

$$I(\theta) = \frac{1}{\beta^2}\left\{\left[\frac{1}{2}\cos 2\beta + \beta \mathrm{Si}(2\beta)\right]\frac{J_1^2(2\alpha)}{\alpha^2} - \frac{1}{(2\alpha)^2}\left[\frac{J_1(4\alpha)}{2\alpha} - 2J_0(4\alpha) - 1\right] + \frac{1}{2}\sum_1^{\infty}(-1)^{n+1}\frac{(2n)!}{(n!)^4}\alpha^{2n-2}\psi_{n-1}\right\}$$

[1] These studies were aided by a contract (NONR 12800) between the Office of Naval Research, Department of the Navy and Syracuse University, and by a contract (NONR 12801) between the Office of Naval Research, Department of the Navy and the State University of New York. Both of these contracts were supported jointly by the Office of Naval Research and the Atomic Energy Commission.

[2] Fellow of the New York State Division of the American Cancer Society during part of these studies.

[3] It is appreciated deeply that Professor Peter Debye and Dr. Fred Bueche of Cornell University developed the theoretical concepts presented here and continued to help in the solving of the technical details of the method.

[4] The relative index of refraction, $m = \frac{n'}{n_0}$, where n' = refractive index of solute particle, n_0 = refractive index of pure solvent. For protein molecules in solution m is close to unity.

where

$$\psi_n \equiv \int_0^1 \frac{1 - (1 - x^2)^n}{x^2} \cos(2\beta x)dx$$

and

$$\mathrm{Si}(2\beta) \equiv \int_0^{2\beta} \frac{\sin u}{u}\, du.$$

Also, the J_0 and J_1 are Bessel functions of zero and first order and

$$\alpha \equiv \frac{2\pi R}{\lambda_m} \sin\frac{\theta}{2}, \qquad \beta \equiv \frac{\pi L}{\lambda_m} \sin\frac{\theta}{2},$$

where λ_m is the wavelength of light in the medium.

These expressions may be expanded in series to give:

EQUATION 2

$$\beta^2 I(\theta) = \left[\tfrac{1}{2}\cos 2\beta + \beta \mathrm{Si}(2\beta)\right] \frac{J_1^2(2\alpha)}{\alpha^2}$$
$$- \frac{1}{(2\alpha)^2}\left[3\frac{J_1(4\alpha)}{2\alpha} - 2J_0(4\alpha) - 1\right] - \frac{\alpha^2 \sin 2\beta}{4\beta}$$
$$+ \frac{5}{24}\alpha^4\left[\frac{\sin 2\beta}{2\beta} - 2\frac{\cos 2\beta}{(2\beta)^2} + 2\frac{\sin 2\beta}{(2\beta)^3}\right]$$
$$- \frac{\alpha^6}{144}\left[\frac{\sin 2\beta}{2\beta} - 2\frac{\cos 2\beta}{(2\beta)^2} - 6\frac{\sin 2\beta}{(2\beta)^3}\right.$$
$$\left. - 24\frac{\cos 2\beta}{(2\beta)^4} + 24\frac{\sin 2\beta}{(2\beta)^5}\right] + \ldots .$$

Equation 2 is not useful for experimentation. The generalized expression has been reduced to a workable form by assuming the fibrin strands had a radius less than about 1,100 Angstroms and a length[5] greater than about 30,000 Angstroms, such that L/λ_m is greater than 10, β is greater than 30, α is less than 1.6 and R/λ_m is less than 0.3. The reduced equation is given by:

EQUATION 3

$$I = \frac{(\text{constant})\ (X)\ (J_1^2(2\alpha))}{\alpha^2},$$

where: I is the intensity of light scattered at angle θ, and

$$X = \frac{1}{\sin\frac{\theta}{2}}.$$

The constant in front may be adjusted at will for normalization purposes.

[5] The length is not clearly defined since the fibrin strands form a three dimensional network. Most likely, L represents the distance from one junction point of three or more strands to the next. Measurements of this distance from electron microscope photographs would give a false low value since the clot is collapsed and false junction points are created.

MATERIALS AND METHODS

Preparation of samples. Fibrinogen was obtained from two sources, bovine Fraction I (Armour) and citrated human plasma (1 part of 0.109 molar sodium citrate plus 9 parts of venous blood) from hospitalized patients and laboratory personnel. Gamma globulin and albumin were from bovine sources (Armour). The bovine plasma fractions were dissolved under vacuum and dialyzed at + 1 C. for 24 hours against phosphate buffers of desired pH and ionic strength. Citrated fresh human plasma usually has a pH of 7.5 to 8.0. If lower pH values were desired, a small amount of 0.1 N HCl was added dropwise with constant agitation. If the protein solutions were diluted, 0.15 molar sodium chloride was always used as a diluent. The protein solutions were clarified by centrifuging 30 minutes at 18,000 G (Sorval) at + 10 to + 20° C. Bovine thrombin (Parke, Davis & Co.) was used to clot both the bovine fibrinogen and human plasma.

Protein analyses. Fibrinogen analysis was performed by the method of Morrison (15). Albumin and globulins were determined by the method of Kibrick (16).

Platelet counts were determined by direct counting using a modified Rees-Ecker diluting fluid (17). pH was measured on a Cambridge Instrument Co. Model R glass electrode pH meter.

Optical density was measured on a model DU Beckman spectrophotometer using 1.0 cm. cells at room temperature, against a 0.15 molar sodium chloride solution blank.

Angular scattering measurements. The intensity of scattered light was measured with a commercial light-scattering microphotometer (American Instrument Co.) in cylindrical, optically good cells with an internal diameter of 34 mm. The light source was a standard mercury vapor tube (AH4) and three filters were routinely used: λ_v = 436, 546, 578 millimicrons ($\lambda_m = \lambda_v/n$, where n is the refractive index). The incident beam was 4 × 8 mm. and a 10-degree solid angle receiver nose piece was used. All readings were multiplied by sin θ to correct for differences in scattering volume. This correction agreed very well with the experimental correction using a dilute fluorescein solution between 60 and 130 degrees. Beyond these limits, the agreement was poor. If no polarizer was used in the incident beam, the observed intensities were also multiplied by $1/1 + \cos^2\theta$ to correct for polarization. This correction factor proved experimentally to be very good when one sample was measured both with and without a polaroid (horizontal axis) in the incident beam. This indicates that no serious depolarization takes place. If other proteins are present besides fibrinogen, and particularly with plasma where there may be a variable amount of lipemia, the solutions will scatter light before

they are clotted. To correct for this, a sample of the solution to be clotted was measured for scattering of exactly the same concentration as that which was clotted. These readings of the unclotted solutions were then subtracted from the readings of the clotted solutions. When fibrinogen alone was used the amount of scattering of the fibrinogen solutions was negligible compared to the scattering of the fibrin clot, in the concentrations used. Small changes of the intensity of the incident beam were determined by measuring the intensity of light at 0 degrees with no cell in the system, before and after each set of readings. Readings were taken between 60 and 130 degrees at 5- or 10-degree intervals. Angles lower than 60 degrees were not used since, as θ becomes small, β becomes small, and the assumption of Equation 3 is that β is moderately large. Also, at the low angles there is a greater amount of secondary scattering which cannot be accounted for by theory. It is to be noted that absolute scattering intensities are not necessary with this method. Only relative values at the different angles are needed.[6]

Method of determining fibrin strand radii from scattering measurements

Using Equation 3, a set of master curves were constructed, plotting IX vs. X^2 for various R/λ_m values, and normalizing them all to one point (we used $X^2 = 1.21$, IX = 20.0, although any convenient point may be used). The corrected experimental intensity readings were then normalized to the same point, graphed, and the R/λ_m value read by comparing with the master curve. Since λ_m is known, R can be computed immediately.

The reason the plot IX *vs.* X^2 was used is as follows: Using Equation 2, a rough approximation to I (θ) may be obtained if one neglects the higher terms in α^3 and $1/\beta^2$. One then obtains:

$$IX = \frac{\pi}{KL}\left\{X^2 - R^2K^2 + 5/12\,\frac{K^4R^4}{X^2} - \frac{2X^2}{\pi KL}\right\},$$

where $K = 2\pi/\lambda_m$.

Unless R is extremely large, the R^4 term may be dropped. Then we obtain:

EQUATION 4

$$IX = AX^2 - BX^2 - C,$$

where $A = \pi/KL$, $B = 2/K^2L^2$, $C = \pi R^2K/L$.

If L is large, Equation 4 may be reduced to

EQUATION 5

$$IX = AX^2 - C.$$

Using Equation 5 and plotting IX vs. X^2 should give a straight line. Approximate values of R can be obtained from this relationship since $R^2 = \lambda_m^2C/40A$. If R is large, there is concave curvature at low values of X^2. If L is small, there is convex curvature at high values of X^2.

From Equation 4, it can be seen that $L = A\lambda_m/B\pi^2$. The use of curvature with the IX vs. X^2 plot to determine L must be with caution since excessive secondary scattering will also give this type of curvature. The curvature is lost (the X^3 term of Equation 4 drops out) when L $\gtrsim$ 10,000 Angstroms at the wavelengths which were used. When this happens L may be calculated if the type of network structure is known. If a cubic lattice is postulated, it can be easily seen by geometry that:

EQUATION 6

$$L^2 = 3\pi R^2d/c,$$

where d is the density (equal approximately to one) and c the fibrin concentration in gms. per cc. Furthermore, the molecular weight of this segment of the fibrin strand can then be calculated:

EQUATION 7

$$M.W. = \pi R^2N_0L/\bar{v},$$

where N_0 is Avogadro's number, $\bar{v}$ is the partial specific volume, = 0.725 (18).

Validity of results. The radii determined on one sample using three different wavelengths usually were within 3% of each other. Possible errors in the interpretation of the results include:

1. Secondary scattering, if marked, will in general cause a false low result. Since the turbidity increases with decreasing wavelength, the lower wavelengths show more secondary scattering.

2. The assumptions used to derive Equation 3 must be met. Thus, if the radius is greater than 1,100–1,300 Angstroms, R/λ_m is no longer less than 0.3. The L/λ_m ratio will be discussed later. Between the range of 18 Angstroms (radius of fibrinogen molecule [19]) to 100 Angstroms, the method is not very sensitive due to the nature of the Bessel function.

3. True absorption of light will interfere with the method. Hemoglobin is the chief offender. An absorption curve was determined for the unclotted sample and, if there was an absorption peak in the region of 410 millimicrons, the sample was discarded.

RESULTS AND DISCUSSION

Any report concerned with fibrin clot measurements must include information about a large number of variables. For the experiments with Fraction I, the following factors were considered important: pH, ionic strength, thrombin concentration, fibrinogen concentration, time that the measurements were taken after adding thrombin, treatment of the fibrinogen in the processing of Fraction I and its resolution, other

[6] Theoretically it is possible to measure the fibrin strand size by determining the total turbidity as a function of wavelength in a spectrophotometer. This approach was attempted but due to the larger number of approximations necessary in the development of the theory and the technical difficulties in separating the primary beam of light from the low angle scattered light, it was abandoned for the simpler and more accurate angular scattering method.

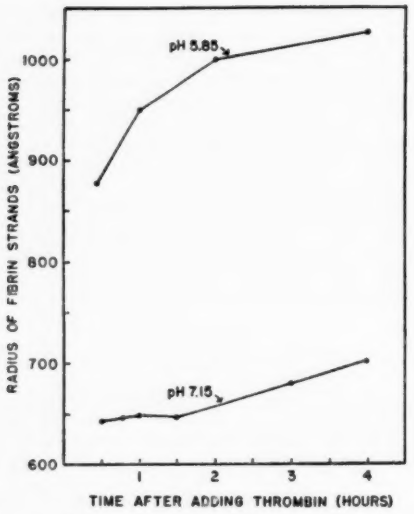

FIG. 1. EFFECT OF pH ON THE RADIUS OF FIBRIN STRANDS AS CALCULATED FROM LIGHT SCATTERING MEASUREMENTS

proteins present, such as the addition of albumin or gamma globulin plus the other proteins besides fibrinogen present in Fraction I. Without entering into the controversial aspects of blood coagulation, one may consider in plasma, in addition to the above factors, the platelet concentration, certain electrolytes (notably calcium), and perhaps substances said to inhibit or accelerate the action of thrombin. A few variations of the above factors are given below in order to illustrate the method. Extension of these experiments with physiological interpretations is in progress.

I. Observations on Light Scattering of Fraction I Clots

A. Effect of pH and time

In all experiments the ionic strength was kept constant at 0.15. The temperature during clotting was always 24 to 26° C. The thrombin concentration was kept constant for both Fraction I and plasma so that the final thrombin concentration was 0.3 units per cc. (15). The proteins other than fibrinogen present in the standard preparation of Fraction I were disregarded. In the first experiment the fibrin strand radii were measured at two different pH values at various times after adding thrombin (Figure 1). The fibrin concentration was that amount of protein which had clotted at one hour after adding thrombin. In the region of pH 7.0 to 7.8 this is always at least 90 per cent of the amount clotted at the end of four hours, both by our experiments and those of other workers (20). At more acid pH values this one hour concentration tends to decrease to about 80 per cent of the four hour value with our fibrinogen and thrombin ranges. In this experiment the one-hour fibrin concentration at both pH values was 0.33 gm. per liter. The optical densities (Figure 2) were taken at 436 millimicrons although the curves at other wavelengths showed similar shapes. The optical density curves in general agreed with recent reports (20, 21) on the kinetics of the fibrinogen-fibrin reaction.

A more detailed examination of the IX vs. X^2 curves at pH 5.85 showed that none had any curvature at 30 minutes. Thus, at this time end-to-end polymerization had proceeded to the point where the fibrin segments were greater than approximately 10,000 Angstroms in length (Equations 4 and 5). However, lateral polymerization continued after 30 minutes of clotting (Figure 1). On the other hand, at pH 7.15, the 30- and 45-minute samples showed curvature at

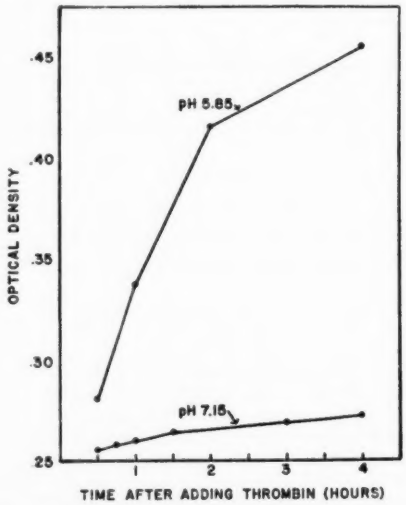

FIG. 2. EFFECT OF pH ON THE OPTICAL DENSITY OF FIBRIN CLOTS

TABLE I

The effect of fibrinogen concentration on the fibrin strand radii

Fibrin conc., gm./L.	pH	R in Å	Fibrin conc., gm./L.	pH	R in Å	Fibrin conc., gm./L.	pH	R in Å	Fibrin conc., gm./L.	pH	R in Å
0.044	7.34	400	0.22	7.11	470	0.37	7.21	520	0.48	7.24	450
0.10	7.28	480	0.28	7.21	460	0.37	7.21	540	0.49	7.24	480
0.12	7.12	600	0.30	7.26	600	0.42	7.21	480	0.49	7.24	440
0.13	7.34	460	0.33	7.15	650	0.44	7.22	600	0.65	7.22	480
0.14	7.20	660	0.33	7.15	640	0.45	7.24	520	0.84	7.21	470
0.16	7.17	550	0.33	7.15	650	0.47	7.24	450			
0.20	7.23	650	0.34	7.20	550	0.47	7.24	460			

high values of X^2 (theta values of about 60 to 75 degrees); after 45 minutes it was absent. Since the optical density changed very little during the 30-minutes to 4-hour range, it may be assumed that the curvature at the earlier times was not due to secondary scattering but to a small value of L. Also, to be noted at pH 7.15, the radius increased only slightly from 30 minutes to 4 hours (Figure 1). This indicated that at this pH the lateral polymerization preceded the end-to-end polymerization. This agrees with the conclusions of Edsall and Lever (20) and does not conflict with Steiner and Laki (14) who have measured the partially polymerized fibrinogen dimensions just before the gel point, whereas these measurements were considerably after gelation.

The value of L of the pH 7.15 sample at one hour calculated by using Equation 6 was 1.1×10^5 Angstroms and the molecular weight of this segment was 1.22×10^{11}. Using the curvature of the IX vs. X^2 plot and Equation 4, the L values at 30 and 45 minutes were found to be about 1,600 and 3,400 Angstroms, respectively. If L is so small, then the conditions used to derive Equation 3 are no longer valid. It should be pointed out that if $R/\lambda_m \cong 0.2$ then L/λ_m may be as small as 3 (instead of greater than 10) and as α gets smaller, the better will be the approximation. These values of L ($L \gtrsim 12{,}000$ Angstroms) are still so low that one may rightly question the value of R. The method of obtaining L using Equation 4 was also an approximation. Therefore, both the R and L values of the 30- and 45-minute samples are in doubt, although they probably represent values in the general order of magnitude of the true ones. For purposes of standardization the following data for the determination of radii were recorded one hour after adding the thrombin. This seems justified if the pH is greater than 7.0.

B. Effect of fibrinogen concentration

The fibrin strand radii with varying fibrinogen concentrations were determined (Table I). Further analysis of the data was more revealing. As mentioned above, the results obtained on any one sample were usually within 3 per cent of each other as judged by agreement at three different wavelengths. If one sample of fibrinogen was clotted in a number of different cells, the coefficient of variation of the measurements was under 7 per cent. However, from one preparation of fibrinogen to another (Table I) the results varied by as much as 40 per cent. For this reason, in determining the effect of changing one variable, the same preparation of fibrinogen was used. In the pH range 7.1 to 7.4, there seemed to be very little dependence of the fibrin strand radius on fibrinogen concentration within the concentration range measured. This is understandable for two reasons:

TABLE II

The effect of heating the fibrinogen solution for 30 minutes at 45° C. before clotting

The pH was 7.21

Unheated		Heated	
Fibrin conc., gm./L.	R in Å	Fibrin conc., gm./L.	R in Å
0.84	470	1.36	350
0.42	480	0.66	330
0.28	460	0.41	330

TABLE III

The effect of adding albumin to the fibrinogen solution

pH	Fibrinogen, gm./L.	No albumin R in Å	Final albumin conc., gm./L.	R in Å	% Increase of R
7.11	0.22	470	65.0	630	34
7.15	0.33	660	106.0	800	21

TABLE IV

The effect of adding gamma globulin to the fibrinogen solution
The pH was 7.22 and the fibrin concentration was 0.65 gm. per liter

Final gamma globulin conc., gm./L.	R in Å
0.0	480
22.2	500
48.8	490
71.0	640
93.3	610

TABLE V

The effect of pH on fibrin strand thickness with fresh human plasma

pH	Fibrin, gm./L.	Albumin, gm./L.	Globulins, gm./L.	Platelets per cu. mm.	R in Å
7.85	0.28	6.4	2.5	1,700	730
7.81	0.18	4.4	3.0	1,400	755
7.34	0.24	3.2	1.8	1,000	820
6.70	0.23	5.9	1.8	1,800	790
5.81	0.22	6.0	1.8	460	880

1) the radius is dependent only on the square root of the concentration (Equation 6), and *2*) L is also changing. If the length decreases enough, it is possible that the radius may decrease with increasing concentrations of fibrin, although this has not been noted experimentally.

C. Effect of heating the fibrinogen solution before clotting

In the next experiment (Table II) half the fibrinogen preparation was heated in a water bath for 30 minutes at 45° C. before adding thrombin. Both samples were at 25° C. when the thrombin was added and the pH of the samples was 7.21. Heating the fibrinogen solution before clotting decreased the fibrin strand thickness rather markedly.

D. Effect of adding albumin

Two experiments were performed in which an albumin solution was added to the fibrinogen solution before adding the thrombin (Table III). In both experiments albumin increased the diameter of the fibrin strands significantly (more than 7 per cent increase).

E. Effect of adding gamma globulin

A similar experiment was performed using gamma globulin instead of albumin. The pH was 7.22, the fibrin concentration 0.65 gms. per liter (Table IV). Gamma globulin increased the fibrin strand thickness somewhat. This agrees with a previous observation (22) which showed there was an increase in the amount of light scattered from fibrin clots when egg globulin was added to the clotting mixture.

II. Observations on Light Scattering of Plasma Clots

The ionic strength, temperature, thrombin concentration (by clotting with added thrombin) and time of measurements after adding thrombin were kept constant at the same values as above with Fraction I. Platelets were kept at a negligible concentration by centrifugation. The calcium ion concentration contributed less than 2 per cent to the total ionic strength.

A. Effect of pH on fresh citrated plasma

From five experiments from five different plasma samples (Table V), it appeared that in general the fibrin strands of fresh plasma were somewhat thicker and were less influenced by pH than Fraction I fibrin.

B. Effect of heating

Part of one sample of fresh plasma was heated for 30 minutes at 45° C. before clotting (Table VI). Heating under these conditions did not decrease the fibrin strand thickness to the marked extent noted with Fraction I fibrinogen.

TABLE VI

The effect of heating fresh plasma for 30 minutes at 45° C. before clotting

	pH	Fibrin, gm./L.	Albumin, gm./L.	Globulins, gm./L.	Platelets per cu. mm.	R in Å
Unheated	7.34	0.24	3.2	1.8	1,000	820
Heated	7.30	0.24	3.2	1.8	860	700

TABLE VII

Fibrin strand radii of aged blood bank plasma
The pH was 7.12 and platelets were absent

Fibrin, gm./L.	Albumin, gm./L.	Globulins, gm./L.	R in Å
0.38	3.4	2.2	320
0.72	6.4	4.2	330

C. *Effect of aging*

One experiment was performed using pooled blood bank plasma which had been stored in the frozen state approximately one year before using. The pH was 7.12 and platelets were absent. The low values (Table VII) were remarkably close to the values obtained with Fraction I which had been heated before clotting (Table II). This suggests that a fibrin strand diameter of 300 to 350 Angstroms is the lowest value possible if gelation occurs.

It should be emphasized that drawing conclusions by comparing experiments on protein fractions with experiments on whole plasma may be misleading. Also, the transition from the fine to coarse type of clot occurs at a different pH range with human fibrinogen than with bovine fibrinogen (20).

SUMMARY

1. A generalized expression for the angular scattering of light by randomly oriented cylindrical rods is given. This general expression has been simplified to apply to cylinders of radii between 100 and 1,300 Angstroms, and lengths greater than 12,000 Angstroms.

2. A few illustrations of the application of this theory to the determination of fibrin strand size are presented.

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BASAL CARDIAC FUNCTION AND BODY COMPOSITION WITH SPECIAL REFERENCE TO OBESITY[1]

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It has been known for many years that the surface area is increased with increasing obesity and that this increase in surface area is accompanied by a proportional increase in basal oxygen consumption (1). Therefore, it has been reasoned that in the presence of obesity cardiac output and cardiac work in the basal state are increased approximately in proportion to the increase in oxygen consumption (2). This reasoning is based on the assumption that the quantity of oxygen carried away by a liter of blood from the lungs (the arteriovenous oxygen difference) is constant in lean and obese individuals.

Certain generally accepted facts support this argument. Thus it is generally agreed that basal cardiac output is proportional to surface area and oxygen consumption. The relationship between surface area and cardiac output has been re-examined in a recent publication from this laboratory (3) and it was found that as a first approximation, the cardiac output is directly proportional to surface area. However, the individual differences in surface area accounted for only 40 per cent of the individual differences in cardiac output. Furthermore, it has been found that while resting cardiac output is proportional to oxygen consumption; the individual differences in oxygen consumption account for only 50 per cent of the observed variability in cardiac output (4). It is clear then that factors other than body size as measured by the surface area or oxygen consumption influence the basal cardiac output. Among the several factors which could account for the unexplained variability is body composition, particularly the fat content of the body. It is known that fat tissue has a metabolism (5) but nothing is known regarding the arteriovenous oxygen difference of blood flowing through fatty tissues or the rate at which it flows.

Precise investigation of the relationships between cardiac function and obesity has been hampered by the inability of the investigator to estimate the relative amounts of fat and other tissue in a given individual. The use of the specific gravity of the body to estimate the relative amount of fat in intact man (6) makes such investigations profitable. It is the purpose of this paper to examine the quantitative relationships between cardiac output, cardiac work and the arteriovenous difference on the one hand and the fat content of the body on the other.

SUBJECTS AND CONDITIONS

The subjects used in this investigation were 34 male university students, 18 to 31 years old, who were free of disease detectable by physical examination. Deliberate efforts were made to obtain a scattering of men from very fat to very lean. The men reported to the laboratory in the morning without breakfast or late in the afternoon without lunch. They rested for one half hour before observations were made. All procedures were carried out in an air-conditioned room. Duplicate determinations of cardiac output were made at one sitting. This was followed by a measurement of the intra-arterial blood pressure. The specific gravity of the body and a roentgenogram of the chest were made after the other cardiovascular measurements had been carried out. In 22 of the 34 subjects, duplicate cardiac output determinations were made on two separate days. Intra-arterial pressures were obtained on 20 subjects and roentgenograms of the chest on 15.

METHODS

The cardiac output measurements were made with the acetylene technic of Grollman. The final calculations were carried out with the corrections worked out by Chapman and his associates (7) to bring the values into

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line with those of the direct Fick (catheter) method. Intra-arterial pressures were measured with a strain gauge and a Cournand needle. The complete recording system was so adjusted that it would pass a square wave of 300 mm. Hg in 0.02 second with a 5 per cent overshoot beyond the base line. Static calibrations were carried out with each determination. The specific gravity of the body was determined from the weight of the individual in water and in air. The volume of residual air in the lung was determined while the men were being weighed under water so that a simultaneous determination of weight under water and residual volume was obtained. The method of Cournand and his co-workers was employed for the measurement of lung volume (8). The details of the weighing procedure have been reported elsewhere (9). In those cases in which measurements of residual volume were not made, a standard correction of 1,500 cc. was applied. The per cent of body weight represented by fat was calculated from tables given by Rathbun and Pace (10). The size of the heart was calculated from measurements of the area of the cardiac shadow on a six-foot roentgenogram of the chest by the method of Keys and his associates (11). The mean arterial pressures were obtained by integrating the recorded pulse wave with a planimeter. The cardiac work was calculated as the mean arterial pressure × the cardiac output, and the kinetic energy component was calculated using the aortic sizes given by Bazett and his group (12). Surface area was calculated according to the procedure of Du Bois.

RESULTS

The data for the individual subjects are presented in Table I. The cardiac outputs in liters

TABLE I

*The anthropometric characteristics and cardiac output along with related measures of cardiovascular function of 34 young men**

Subject	Age	Height	Weight	S. A.	% Fat	Fat	L. B. M.	Cardiac output	A-V	B. P.	Cardiac vol.	Resid. lung vol.
Nay	28	165	53.68	1.58	17.5	9.4	44.6	4.08 (4)	53	89	375	1.001
CVD 56	24	169	51.80	1.58	5.0	2.6	49.2	3.79 (2)	53			
Eric	20	178	54.52	1.69	8.4	4.6	49.9	5.02 (4)	43	76	492	1.866
CVD 104	23	162	53.10	1.56	1.3	0.7	52.4	5.23 (2)	44			
Scan.	19	182	53.73	1.72	1.8	1.0	52.8	5.63 (3)	34	62	524	2.055
Alt.	20	169	71.93	1.83	22.0	15.8	56.1	4.60 (4)	43	83	479	1.296
Will.	20	176	62.00	1.73	7.4	4.6	57.4	5.99 (4)	34	68	583	1.727
CVD 3	25	179	58.60	1.74	1.4	0.8	57.8	5.63 (2)	37			
Koch.	24	175	76.40	1.92	24.0	18.3	58.1	4.72 (4)	46	101		1.115
CVD 7	25	167	67.00	1.74	11.2	7.5	59.5	4.15 (2)	53			
Ott.	31	173	86.00	2.02	29.6	25.5	60.5	4.48 (4)	51	87	410	1.118
Ort.	20	166	80.15	1.89	24.0	19.2	60.9	4.78 (4)	52	72	674	0.980
Lin.	21	175	62.20	1.75	0.4	0.2	62.0	4.92 (2)	42			
CVD 17	22	181	69.20	1.88	9.3	6.4	62.8	5.94 (2)	37			
Traut	22	179	69.60	1.87	8.8	6.1	63.5	5.90 (4)	38	97	574	1.191
Torg.	24	178	75.60	1.94	15.6	11.8	63.8	5.68 (4)	47	87		1.283
Fors.	29	176	65.20	1.80	1.8	1.2	64.0	5.41 (4)	39	90	615	2.498
Stra.	21	171	72.10	1.84	10.7	7.7	64.4	6.08 (4)	40	100	727	1.125
Podem.	23	173	87.27	2.04	26.0	22.7	64.6	4.93 (4)	46	80	478	1.182
CVD 120	24	176	79.32	1.94	18.5	14.7	64.6	5.88 (2)	43			
CVD 119	20	177	71.80	1.89	9.8	7.0	64.8	6.03 (2)	37			
CVD 101	21	185	70.60	1.92	7.0	4.9	65.7	5.50 (2)	44			
CVD 162	24	181	73.70	1.93	10.8	8.0	65.7	5.48 (4)	45	81	852	
Donal.	25	184	70.40	1.92	6.9	4.9	65.5	5.87 (4)	41	86		1.216
CVD 41	25	170	76.50	1.87	13.6	10.4	66.1	4.40 (2)	46			
Svie.	25	179	77.40	1.96	14.6	11.3	66.1	6.88 (4)	37	100		1.368
CVD 13	22	177	70.10	1.86	3.7	2.6	67.5	5.58 (2)	40			
CVD 110	20	175	87.57	2.00	22.5	19.7	67.9	5.83 (4)	41			
Adam.	23	172	76.20	1.89	8.8	6.7	69.5	6.43 (4)	34	101	548	1.389
CVD 31	26	175	95.30	2.10	24.5	23.3	72.0	6.64 (2)	38			
Erwi.	27	184	101.43	2.23	26.5	26.9	74.6	5.83 (4)	41			1.980
Teit.	19	189	84.40	2.10	7.4	6.2	78.2	6.52 (4)	40	92		1.319
Chri.	21	179	84.00	2.02	4.1	3.4	80.6	7.22 (4)	31	91		1.619
Duc.	24	184	132.50	2.53	33.7	44.6	87.8	7.35 (4)	41	93	642	1.361
Mean	23.1	175.9	74.16	1.89	12.90	10.61	63.56	5.54	42.1			
S.D.	2.91	6.2	15.87	0.19	9.22	9.73	8.70	0.87	5.8			

* Abbreviations and units: Age, in years; Height, in cm.; Weight, wt. of whole body in kg.; S.A., surface area in square meters; % Fat, fat as per cent of whole body weight; Fat, weight in kgs.; L. B. M., lean body mass (body weight minus weight of fat) in kg.; Cardiac output, liters per minute, the number in parenthesis refers to the number of C. O. determinations that were performed; A–V, the arteriovenous oxygen difference in cc. per liter of blood; B.P., the mean intra-arterial blood pressure in mm. of Hg.; Cardiac vol., the volume of the heart in cc.; Resid. lung vol., the residual volume of the lungs in cc.

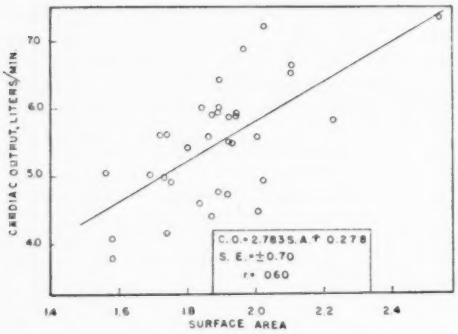

FIG. 1. THE RELATIONSHIP BETWEEN CARDIAC OUTPUT IN LITERS PER MINUTE AND SURFACE AREA IN SQUARE METERS IN 34 HEALTHY YOUNG MEN

per minute are the mean values of the designated number of determinations. The values for the weight of lean body mass were obtained by subtracting the weight of fat from the total body weight. Since the constants for converting specific gravity to per cent of fat were derived from the weight of fat extracted by fat solvents from guinea pig carcasses (10) this value is equivalent to the lean tissue mass and theoretically includes any water and salt which may be present in the fatty tissues. In addition, the calculated value of fat is subject to such errors as may arise from assuming a constant specific gravity for the lean body mass.

Cardiac output and body composition

The correlations between important cardiovascular characteristics and the various measures of body size and composition have been calculated and are presented in Table II, along with other pertinent correlations. Significant correlations were found between cardiac output and body weight, surface area, and lean body mass, but no significant relationship was demonstrated between cardiac output and weight of body fat or per cent of body fat.

The data relating cardiac output to surface area and lean body mass are plotted in Figures 1 and 2. The regression equations relating cardiac output with these two variables are given below:

Equation 1. C. O. = 2.783 S. A. + 0.278
Equation 2. C. O. = 0.074 (L. B. M.) + 0.838,

where cardiac output (C. O.) is expressed as liters

TABLE II

Coefficients of correlation (r) between cardiac output in liters per minute, arteriovenous oxygen difference (A–V) in cc. per liter, cardiac work in kilogram meters per minute, total peripheral resistance (T.P.R.) in dynes cm.$^{-5}$ seconds and the several measures of body size and composition

Measurement	No. of subjects	Body weight	Surface area	Per cent fat	Fat, Kg.	Lean body mass, Kg.
Cardiac output	34	0.52	0.60	−0.01	0.16	0.75
A–V	34	0.17	−0.22	0.35	0.22	−0.43
Cardiac work	20	0.50	0.55	−0.13	0.07	0.76
T.P.R.	20	0.09	0.18	0.27	0.07	−0.22

For 34 subjects r is significantly different from zero at the 5% level of probability if $r = 0.34$ and at the 1% level if $r = 0.44$. For 20 subjects similar values of r are 0.44 and 0.56, respectively.

per minute, surface area (S. A.) in square meters, and lean body mass (L. B. M.) in kilograms. The standard error of estimate of Equation 1 is 0.70 liters per minute, and that of Equation 2 is 0.58. The basal cardiac output can therefore be predicted most accurately from the lean body mass.

Only a small advantage in prediction was found when multiple regression equations were used. The two most successful combinations are presented below:

Equation 3. C. O. = 0.061 (L. B. M.) + 0.034 height − 4.313
Equation 4. C. O. = 0.084 (L. B. M.) − 0.02 (fat weight) + 0.415,

where height is expressed in centimeters and fat and lean body mass in kilograms.

For Equation 3, the coefficient of multiple cor-

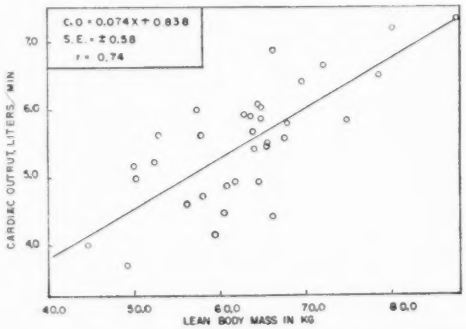

FIG. 2. THE RELATIONSHIP BETWEEN CARDIAC OUTPUT IN LITERS PER MINUTE AND LEAN BODY MASS IN KILOGRAMS IN 34 HEALTHY YOUNG MEN

relation relating the predicted cardiac output with the observed was found to be 0.77 while that for Equation 4 was 0.78. The standard errors of estimate when rounded off to two places were 0.55 liters per minute for both equations.

It will be noted that Equation 2 assumes a linear relationship between the weight of a part of the body and cardiac output. However, it is known that total body weight raised to the 3/4 power (a curvilinear relationship) is more closely related to oxygen consumption than is the linear regression equation (13). It is of interest then to examine the relationship of cardiac output and lean body mass raised to the power which provides the best fit. The regression equation between the log of the cardiac output and the log of the lean body mass yields the following power equation:

Equation 5. $C.O. = 0.167 (L.B.M.)^{0.848}$.

The coefficient of correlation relating the logarithms of cardiac output and lean body mass was found to be 0.73 or of the same order of magnitude as that found in the linear relationship. It appears then that there is no special advantage in the power equation over the linear equation.

The arteriovenous oxygen difference and body composition.

The coefficients of correlation between the arteriovenous oxygen difference and the various measures of body size and composition are included in Table II. There are two correlation coefficients which are significantly different from zero. A positive correlation coefficient of 0.35 ($p < 0.05$) between arteriovenous oxygen difference and percentage of fat in the body suggests a specific effect of fat. A negative correlation between the arteriovenous oxygen difference and lean body mass of -0.43 ($p < 0.02$) indicates that the lean body mass is a more important determining factor. Neither of these relationships are good enough to suggest that they be used as reference points for predicting arteriovenous oxygen differences. However, we may examine multiple regression equations and here we find more useful correlations.

The relationships between cardiac output and measures of fat and lean body mass are given below:

Equation 6. $A\text{-}V\,O_2 = 61.73 + 0.321$ (per cent of fat) $- 0.374$ (L. B. M.)

Equation 7. $A\text{-}V\,O_2 = 66.52 + 0.310$ (wt. of fat) $- 0.436$ (L. B. M.),

where all weights are expressed in kilograms, the percentage of fat is the percentage of fat in the total body and the arteriovenous oxygen difference is in cc. of oxygen per liter of blood. The correlation coefficient relating the predicted and observed values of arteriovenous oxygen difference for Equation 6 is 0.65 and for Equation 7 is 0.63 whereas the standard errors of estimates are 0.78 and 0.80 cc. of oxygen per liter of blood, respectively. Both these correlations are significantly different from zero at the 1 per cent level. They are of the same order of magnitude as the correlation coefficients relating surface area and cardiac output, which range between 0.5 and 0.6 (3) and must be considered to have real biological meaning.

Cardiac work and peripheral resistance

Data from which basal cardiac work and total peripheral resistance could be calculated were obtained on 20 men. The means and standard deviations of the cardiac work and total peripheral resistance along with the figures for the several measures of body size and composition are presented in Table III while the correlation coefficients have been given in Table II. It is apparent that the total peripheral resistance is unrelated to any measure of body size or body composition. On the other hand, basal cardiac work is correlated with total body weight, surface area, and lean body mass. As in the case of the cardiac out-

TABLE III

The means and standard deviations of the cardiac work in kilogram meters per minute, the total peripheral resistance in dynes cm.$^{-5}$ seconds, and the several items of body size and composition in 20 young men

	Cardiac work	T.P.R.	Body wt., Kg.	S.A.	Per cent fat	Fat, Kg.	Lean body mass, Kg.
Mean	6.52	1,282.4	75.34	1.91	14.19	11.65	63.70
S.D.	1.57	236.6	16.93	0.19	9.44	10.45	10.12

TABLE IV

The values of the regression coefficients and the standard errors of estimates of the regression equation relating cardiac work in kilogram meters per minute to measurements of body size and composition

	Body weight	Surface area	Per cent fat	Fat, Kg.	Lean body mass, Kg.
b*	0.046	4.44	−0.02	0.01	0.12
a	3.035	−1.97	6.84	6.40	−1.01
S.E.	1.361	1.31	1.55	1.57	1.02

* a and b are defined by the following equation:

$$y = a + bx,$$

where y is the predicted value and x is the reference point.

put, the highest correlation was found between lean body mass and cardiac work. The regression coefficients from which one may construct the regression equations relating cardiac work with the several measures of body size and composition are presented in Table IV.

Estimates of the volume of the heart were obtained in 16 subjects. The relationships between cardiac work per cc. of heart volume and body composition were examined. The largest correlation coefficient found was 0.42 which existed between the percentage of fat in the body and the cardiac work per cc. of heart volume. But this value of r would have to reach 0.50 ($p = 0.05$) before it could be considered significant. The relationships between heart volume and body composition will be discussed elsewhere.

Anthropometric types and cardiovascular functions

Equations 6 and 7 make it clear that different proportions of fat and lean body mass will have definite consequences in cardiovascular function. To illustrate these effects in more detail we have plotted the weight of fat against the lean body mass in Figure 3. The vertical line on the figure indicates the mean of the lean body mass in the group under study and the horizontal line is drawn through the mean of fat weights. The box in the center outlined by the dotted lines encloses an arbitrarily chosen "normal" group. The group in the lower left-hand quadrant is considered to to be low in lean body mass and low in fat; the group in the upper left-hand quadrant, high in fat and low in lean body mass; the group in the upper right-hand quadrant, high in fat and in lean body mass; whereas the group in the lower right-hand quadrant is high in lean body mass and low in fat. The characteristics of the five groups are presented in Table V. Inspection of the data reveals that those persons with a small lean body mass and a large weight of fat have high arteriovenous oxygen differences and those with a large lean body mass and only small amounts of fat have low arteriovenous oxygen differences.

The application of the usual t test demonstrated that the mean arteriovenous oxygen differences were significantly different at the 5 per cent level for the following two pairs: *1*) "normal" vs. high fat, low lean body mass, *2*) high fat, low lean body mass vs. high fat, high lean body mass. In addition, a significant difference at the 1 per cent level was found between the means of the arteriovenous oxygen differences in the following pair: *1*) high fat, low lean body mass vs. low fat, high lean body mass. The differences between the cardiac indexes were also found to be significant between the same pairs of groups. However, in the case of this variable it was shown that the cardiac index of the group with high fat, low lean body mass differed significantly from that with low fat, low lean body mass. None of the groups showed any significant differences in the cardiac output per kilogram of lean body mass.

Other methods of defining anthropometric categories may be employed. We have plotted the weight of fat per unit of height against the lean body mass per unit of height. The means of each

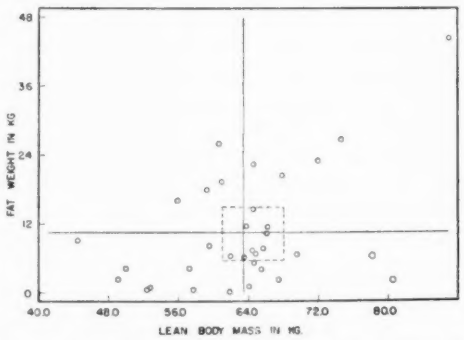

FIG. 3. THE RELATIONSHIP BETWEEN THE WEIGHT OF FAT AND THE LEAN BODY MASS

The horizontal line is the mean of the fat weights and the vertical line is the mean of the fat-free weights. The box in the center encloses the "normal" group.

TABLE V

The means and standard deviations of the arteriovenous oxygen difference (A–V), the cardiac index (cardiac output per square meter of body surface) and the cardiac output in cc. per minute per kilogram of lean body mass along with body composition and anthropometric characteristics of five groups of differing body composition

	Normal	Anthropometric type			
		Fat wt. low L. B. M. low	Fat wt. high L. B. M. low	Fat wt. high L. B. M. high	Fat wt. low L. B. M. high
N	9	8	4	5	7
Age	23.0 ± 1.8	22.1 ± 2.4	23.8 ±5.2	24.0 ± 2.7	23.1 ± 3.6
Wt. of fat	9.3 ± 2.9	2.8 ± 2.6	19.7 ±4.1	27.4 ± 9.9	4.3 ± 2.0
Lean body mass	64.6 ± 1.2	55.1 ± 4.7	58.9 ±2.2	73.4 ± 8.9	70.1 ± 6.6
% standard wt.	107.1 ± 7.9	87.6 ±10.3	121.0 ±9.45	142.1 ±19.3	104.2 ±10.8
A–V	41.08± 4.08	42.57± 7.44	48.0 ±4.48	41.31± 2.62	38.42± 4.27
Cardiac index	3.05± 0.32	2.98± 0.42	2.43±0.15	2.8 ± 0.30	3.14± 0.25
C. O., cc./Kg. L. B. M.	89.9 ±10.2	91.5 ±14.5	78.8 ±3.6	83.2 ± 6.4	86.6 ± 3.9

measurement were used in a manner similar to that in Figure 3 to establish the quadrants. A normal group was chosen and the differences between the mean arteriovenous oxygen differences of the several groups were tested with the *t* test. Significant differences were found between the same pairs of groups which are listed above. In addition, the "normal" group showed a mean arteriovenous oxygen difference which was significantly (5% level) higher than the group which was characterized by a large lean body mass per unit height and small fat weight per unit height.

Anthropometric groups were also defined by plotting the percentage of standard weight for age and height against the percentage of fat, a procedure employed by McCance and Widdowson (14). The quadrants were defined by the line following 100 per cent of standard weight and 9.8 per cent fat. The figure of 9.8 per cent fat is the percentage of fat of a man 22 years old where weight is standard for height and age (15). An alternative presentation involved plotting the percentage of standard weight against the percentage of standard fat for age and height. The latter figure was found by the use of the tables provided by Brozek (16). Both of these procedures allocate the men into groups whose members were: *1*) underweight for height and with a less than normal percentage of fat, *2*) overweight for height and with a greater than normal percentage of fat, and *3*) overweight for height and with a less than normal percentage of fat. In each formulation a control group, clustered around the center of the diagram, was chosen. In each case, it was possible to show that the group which was fat and overweight had an arteriovenous oxygen difference which was significantly larger than the group which was overweight and lean.

DISCUSSION

The classification of body build used here depends entirely on the validity of the specific gravity method. The calculation of the percentage of body fat in an individual depends on knowing the density of the lean body mass. We have used the figure of 1.10, provided by Rathbun and Pace (10). The use of a single figure for the density of the lean body mass is obviously an approximation. Since bone has a density of 1.43 and all other tissues a density of the order of 1.066, the important source of error is the variation in the fraction of bone in the lean body mass. The important relations are given in the following equation:

Equation 8. Per cent of fat

$$= 100\left[\frac{1}{D}\left(\frac{4.188}{0.6334 + R}\right) - \left(\frac{4.562}{0.6334 + R}\right) + 1\right],$$

where D is the observed density of the body and R is the fraction of wet bone in the lean body mass. A density of 1.10 for the lean body mass requires an R value of 0.1214. In a muscular man, who has a smaller value of R, the standard equation will overestimate the fat content of the body, whereas in the thin man, who has a value of R larger than 0.1214, the fat content will be underestimated. Reasonable estimates of the variation in the value of R indicates that the error due to this source is probably not greater than 3 to 4 per cent of the total body weight. This problem will be discussed in more detail elsewhere.

It has been recognized by other investigators (17) that the lean body mass may be a better reference point than surface area for such biologically important functions as basal metabolism, blood volume, etc. This possibility has been confirmed by this investigation in the case of the cardiac output.

However, the advantage of the lean body mass over the surface area as a reference point in clinically normal men appears to depend entirely on the relationships between the arteriovenous oxygen difference and the body compartments. It is not the purpose of this paper to examine the relationships between the basal metabolic rate and the various body compartments. However, it should be mentioned that in this data the correlation between oxygen consumption and surface area was of the same order of magnitude as that between oxygen consumption and lean body mass.

No relationship was found between blood pressure measured intra-arterially and body size or composition. This is, of course, contrary to observations made on large groups using the sphygmomanometer (18) where it was shown that the blood pressure increased with increasing relative weight. It has been assumed that the blood pressure increased with fatness. Our failure to find an increase in blood pressure with obesity is not surprising in view of the recent demonstration that the cuff method overestimates the blood pressure in arms of large diameter and underestimates the blood pressure in arms of small diameter (19, 20). The group studied here is small and it is not possible to state that there is not a relation between blood pressure and degree of obesity. However, it is clear that if such a relation exists the increment of blood pressure per unit of fatness must be small.

Equation 7 makes it clear that certain anthropometric types have characteristic arteriovenous oxygen differences. Since the cardiac output is equal to the oxygen consumption divided by the arteriovenous difference, it follows that the basal cardiac output is not strictly proportional to the basal oxygen consumption in individuals with different body compositions. In addition, the significant differences between the cardiac indexes of different anthropometric groups presented in Table V demonstrates that the cardiac output is not strictly proportional to the surface area in the several anthropometric types.

It is apparent that the largest cardiac outputs per square meter of surface area and per cc. of oxygen consumption will be found in muscular (large lean body mass) individuals who have a relatively small fat content. Fatness *per se* does not necessarily mean that the basal cardiac output and cardiac work has been increased in proportion to the increase in surface area or oxygen consumption. Thus, those people with a relatively small lean body mass and a relatively large mass of fat will have cardiac outputs which are definitely less than that predicted from surface area or oxygen consumption. The person who has a large fat-mass which is superimposed on a large muscle-mass (lean body mass) may have a cardiac output nearly proportional to the surface area and to oxygen consumption.

The increasing arteriovenous oxygen difference associated with increasing amounts of fat on the body may well act as a protective mechanism since it reduces the cardiac work load below that which would exist if the cardiac output always remained proportional to the oxygen consumption. In this connection it is interesting to note that Smith and Willius (21) were able to find evidence of cardiac hypertrophy at autopsy in only 30 per cent of 52 obese patients who were selected because there was no evidence of heart failure or hypertension.

Finally, it should be kept in mind that the conclusions of this investigation apply strictly to young men in their twenties who are living in a university environment. Preliminary studies of middle-aged business men (22) have demonstrated that the arteriovenous oxygen difference changes markedly with age.

SUMMARY AND CONCLUSIONS

1. The basal cardiac outputs of 34 healthy male university students were determined by the acetylene technic and the values were corrected to direct Fick levels. The specific gravity of the body was determined by the water immersion method and the amount of body fat was estimated for all men.

2. It was found that the basal cardiac output was related to body weight by a correlation coefficient (r) of 0.54, to surface area by an r of 0.60, to the weight of lean body mass by an r of 0.74 and finally to the weight of fat by an r of 0.16.

3. Intra-arterial blood pressure was measured

on 20 of these men and basal cardiac work was calculated. The basal cardiac work was related to anthropometric measures of body mass and compartments in roughly the same manner as basal cardiac output. The coefficients of correlation relating basal cardiac work to body weight, surface area, weight of fat-free tissue and fat were found to be 0.50, 0.55, 0.76, and 0.07, respectively.

4. The arteriovenous oxygen difference was positively correlated with fat and negatively correlated with lean body mass. The arteriovenous oxygen difference predicted by a multiple regression equation using the weight of fat and the lean body mass as predictors was related to the observed arteriovenous oxygen difference by a coefficient of correlation of 0.63.

5. It was shown that the basal cardiac output was not strictly proportional to surface area and oxygen consumption in certain anthropometric types.

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FAILURE OF DESOXYCORTICOSTERONE GLUCOSIDE TO ALTER CEREBRAL VENOUS SUGAR CONCENTRATION IN MAN [1]

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Intravenous administration of desoxycorticosterone glucoside (DCG) in man has been reported to cause an immediate liberation of sugar from the brain into cerebral venous blood (1). It is said that a portion of this reducing substance may be galactose rather than glucose as it is non-fermentable (1). The finding in the fasting state of blood glucose levels higher in blood draining an organ than in the blood entering it is unusual except in the liver. As the reported findings postulated a new physiological concept, an attempt was made to confirm these studies.

In the original report, the rise in sugar concentration in internal jugular blood was found after the injection of DCG and during the period of inhalation of 15 per cent N_2O. As these procedures were reported not to cause a change in cerebral blood flow or cerebral oxygen consumption (1), only measurements of blood sugar content were made following *1*) intravenous DCG and *2*) inhalation of 15 per cent and 50 per cent N_2O.

METHOD

Seven physically normal subjects were studied in the fasting state. Blood specimens were obtained from the femoral artery and from the internal jugular vein (2). After control specimens were taken, DCG [3] (50 mg. in 5 cc. water containing 10% glucose and 10% acetamide) was given intravenously into an antecubital vein over a time period of one minute. Simultaneously drawn arterial and cerebral venous blood samples were taken at frequent intervals from one to fifteen minutes after injection.

All blood sugar determinations were done in duplicate, using Nelson's (3) modification of the Somogyi method for measuring blood glucose.

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[3] The desoxycorticosterone glucoside used in this study was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, New Jersey.

RESULTS

In Table I are given the simultaneous arterial-cerebral venous sugar differences in six subjects after intravenous administration of 50 mg. of DCG. No change in A-V difference or in blood sugar level occurred.

Table II shows the effect of 15 per cent and 50 per cent N_2O on blood sugar levels and on arterial-cerebral venous sugar difference. The results are those obtained on one subject after inhalation of 15 per cent N_2O over a period of 10 minutes and, after inhalation of 50 per cent N_2O over a second 10-minute period. The patient noted slight drowsiness with the second procedure but no other symptoms.

DISCUSSION

DCG, in the dosage used, did not significantly change blood sugar levels or arterial-cerebral venous blood sugar difference. The slight immediate, but brief, increase in both arterial and cerebral

TABLE I

Effect of intravenous administration of 50 mg. of desoxycorticosterone glucoside on arterial-cerebral venous glucose differences

(All values are given in mg. %)

	Control	Time in minutes (after DGG)					
		2	4	6	8	10	15
J. S.	10.9	13.1	14.0	12.6		16.1	15.2
D. B.	13.7	13.0	13.2	12.6	12.0	9.0	
B. V.	11.7	13.3	16.9	14.3		14.9	
S. S.	12.0	15.9	12.3	12.3		10.6	
N. P.	12.0	10.0	8.7	8.0	10.7		12.3
B. S.	11.3	13.5	13.9	11.3	10.6		
Mean	11.9	13.1	13.2	11.9		12.4	
Avg. art. blood sugar, mg. %	96.1	98.1	96.2	94.0		93.6	
Avg. cerebral venous blood sugar, mg.	84.0	85.0	83.0	82.1		81.2	

TABLE II

Effect in one subject of 15% and 50% N_2O on arterial-cerebral venous glucose differences
(All values are given in mg. %)

Minutes	Before N_2O		During 15% N_2O breathing									
	−4	−2	1	2	3	4	5	6	7	8	9	10
1) Arterial, mg. %	118.3	117.7		118.8		117.7			116.7			112.1
2) Cerebral venous	109.6	109.6		108.5		106.0			102.9			102.9
3) A–V sugar difference	8.7	8.1		10.3		11.7			13.8			9.2
Minutes	Before N_2O		During 50% N_2O breathing									
	−4	−2	1	2	3	4	5	6	7	8	9	10
1) Arterial, mg. %	108.5	106.5	105.0	103.4	103.9			106.0				103.4
2) Cerebral venous	95.7	96.3	95.7	96.8	96.8			98.8				96.8
3) A–V sugar difference	12.8	10.2	9.3	6.6	7.1			7.2				6.6

venous glucose blood levels is related to the glucose content used as a solvent for the DCG. This amounts to 500 mg. of glucose (5 cc. of DCG is dissolved in 10 per cent glucose and 10 per cent acetamide). This amount would be rapidly dispersed into body tissues and the degree of blood sugar rise in the blood stream would be related to amount of sugar injected, rate of injection, and the time following injection. If all injected glucose remained intravascularly, 500 mg. would theoretically raise the glucose level about 10 mg. per cent in a man of average size.

SUMMARY AND CONCLUSIONS

1. Desoxycorticosterone glucoside, in the dosage used, did not narrow arterial-cerebral venous glucose difference. In no instance did cerebral venous blood sugar content surpass or approach arterial levels.

2. Nitrous oxide in 15 per cent and 50 per cent concentrations, in one patient, did not cause an increase in the cerebral venous blood glucose content.

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BACTERIOLOGY OF THE HUMAN LIVER

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Attention has recently been focused upon the potential role played by the intestinal flora in hepatic disease. The experiments of Gyorgy and his co-workers (1, 2) have pointed to a possible relationship existing between intestinal bacteria and the production of massive hepatic necrosis in rats. With the addition of aureomycin and other antibiotics to the diet of such animals, these investigators have shown that survival is possible under conditions which will otherwise produce a diffuse hemorrhagic necrosis of the liver. A similar phenomenon may explain, at least in part, the improvement noted in certain cases of chronic hepatic disease who have been treated with antibiotics (3, 4). Dramatic but temporary improvement has also been observed with the use of aureomycin administered intravenously in cases of hepatic coma (5). Whether the improvement noted in such cases can be related specifically to an antibiotic effect remains subject to final proof.

Current interest in the bacteriology of the liver has been aroused by Rienhoff (6) and others who propose ligation of the hepatic artery in man for the relief of portal hypertension and bleeding esophageal varices. In dogs such a procedure is known to be highly fatal (7). Markowitz (8) has recently demonstrated, however, that death from the interruption of arterial blood flow to the liver in dogs can be prevented in most cases with the use of penicillin administered immediately postoperatively. This is thought to be related to the prevention of spread of gas bacillus infection in the liver which otherwise ensues in such preparations when the antibiotic is omitted (7, 9).

It has been conclusively demonstrated that bacteria, especially of the genus *clostridia*, are regularly present in the livers of dogs (10–14). Experimental studies in these and other animals have suggested that intestinal bacteria present in the liver and other tissues may be of importance in the pathogenesis of shock and radiation injury (15, 16). Because of these animal experiments and the known function of the liver as the significant filtering organ for portal blood, it seemed of some importance to ascertain whether or not viable bacteria are present in the human liver. If bacteria were demonstrated to be consistently present, conclusions from animal experiments in these important areas of trauma would be more directly applicable to human situations.

Mason and Hart (17) have demonstrated anaerobic organisms in five human livers studied post mortem. This observation is not particularly surprising since it is believed that bacteria of all types may invade the blood stream and the tissues after death. The principal source of such organisms is probably the intestinal tract from which anaerobes reaching the liver could easily originate. Romieu and Brunschwig (18) have recently reported a series of twelve human patients from whom liver biopsies were taken and cultured at the time of laparotomy for neoplasms of the lower gastrointestinal tract. In one of these a positive culture of *Bacillus pyocyaneus* was found not only in the liver but also in the urine and in the operative wound. From this study the authors concluded that there is no "normal flora" of the human liver.

The present investigation was undertaken to determine the possible presence of living bacteria in the human liver in normal persons and in acute and chronic liver disease. It was believed that if the liver is capable of a bactericidal function in life, this bactericidal function was more likely to be impaired when other structural and functional impairment of the liver coexisted. Positive results in such a study would provide a firmer

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basis than now exists for the use of antibiotic therapy in such patients.

EXPERIMENTAL METHODS

All of the specimens used in this study were obtained by means of a biopsy of the liver with a Vim-Silverman needle. Eighty-six samples were obtained from 66 patients, some of the patients having been biopsied on more than one occasion (Table I). Twenty-five of the specimens were obtained with the Silverman needle at the time of laparotomy; eleven from the right lobe and fourteen from the left lobe. All the others were from the right lobe via the transpleural approach. Liver biopsies were also taken from twenty anesthetized dogs (Table II).

A sterile technic was employed in all instances. In the non-laparotomized humans and in the dogs, the biopsy site was thoroughly cleansed and painted twice with merthiolate. The area was then draped and a stab wound made after infiltration with one per cent novocaine (novocaine was not used in the anesthetized dogs). When the specimen of liver was obtained a small piece was immediately removed and planted in 20 cc. of broth prepared according to the formula of Kämmerer and Miller (19).[4] The liver specimens were macerated against the bottom of the tube with a sterile blunt glass rod, and the

[4] This media was used as a primary one in this study since it was experimentally shown that it would support the growth of certain intestinal anaerobic flora not supported by Brewer's thioglycollate media.

TABLE I

Bacteriological studies of human liver biopsies

	Diagnosis	Cultures		Remarks
		Aerobic	Anaerobic	
Case 1	Acute hepatitis	Negative	Negative	
Case 2	Acute hepatitis	Negative	Negative	
Case 3	Acute hepatitis	Negative	Negative	
Case 4	Acute hepatitis	Negative	Negative	
Case 4	Acute hepatitis	Negative	Negative	Biopsy 10 days later
Case 4	Acute hepatitis	Negative	Negative	Biopsy 6 months later
Case 5	Acute hepatitis	Negative	Negative	
Case 5	Acute hepatitis	Negative	Negative	Biopsy 30 days later
Case 6	Acute hepatitis	Negative	Negative	
Case 6	Acute hepatitis	Negative	Negative	Biopsy 30 days later
Case 7	Acute hepatitis	Negative	Negative	
Case 8	Acute hepatitis	Negative	Negative	
Case 9	Acute hepatitis	Negative	Negative	
Case 10	Acute hepatitis	Negative	Negative	
Case 11	Acute hepatitis	Negative	Negative	
Case 12	Acute hepatitis	Negative	Negative	
Case 13	Acute hepatitis	Negative	Negative	
Case 14	Acute hepatitis	Negative	Negative	
Case 15	Acute hepatitis	Negative	Negative	
Case 16	Acute hepatitis	Negative	Negative	
Case 17	Acute hepatitis	Negative	Negative	
Case 18	Acute hepatitis	Negative	Negative	
Case 19	Acute hepatitis	Negative	Negative	
Case 20	Chronic hepatitis	Negative	Negative	
Case 21	Chronic hepatitis	Negative	Negative	
Case 21	Chronic hepatitis	Negative	Negative	Biopsy 9 weeks later
Case 22	Chronic hepatitis	Negative	Negative	
Case 23	Chronic hepatitis	Negative	Negative	
Case 24	Chronic hepatitis	Negative	Negative	
Case 25	Chronic hepatitis	Negative	Negative	
Case 26	Chronic hepatitis	Negative	Negative	
Case 27	Chronic hepatitis	Negative	Negative	
Case 28	Chronic hepatitis	Negative	Negative	
Case 29	Hepatic cirrhosis	Negative	Negative	
Case 30	Hepatic cirrhosis	Negative	Negative	
Case 30	Hepatic cirrhosis	Negative	Negative	Biopsy 6 weeks later
Case 31	Hepatic cirrhosis	Negative	Negative	
Case 32	Hepatic cirrhosis	Negative	Negative	
Case 32	Hepatic cirrhosis	Negative	Negative	Biopsy 8 weeks later
Case 33	Hepatic cirrhosis	Negative	Negative	
Case 34	Hepatic cirrhosis	Negative	Negative	
Case 35	Hepatic cirrhosis	Negative	Negative	
Case 35	Hepatic cirrhosis	Gram neg. bacillus*	Gram neg. bacillus*	Left lobe at laparotomy
Case 36	Hepatic cirrhosis	Negative	Negative	

TABLE I—*Continued*

	Diagnosis	Cultures		Remarks
		Aerobic	Anaerobic	
Case 37	Hepatic cirrhosis	Fungus at 3 weeks†		Left lobe at laparotomy
Case 38	Hepatic cirrhosis	*Strep. pyogenes, E. coli, P. vulgaris & P. mirabilis*		Biopsy 30 minutes after death
Case 39	Hepatic cirrhosis	Negative	Negative	
Case 40	Hepatic cirrhosis	Negative	Negative	
Case 41	Hepatic cirrhosis	Negative	Negative	Right lobe at laparotomy
Case 41	Hepatic cirrhosis	Negative	Negative	Left lobe at laparotomy
Case 42	Hepatic cirrhosis	Negative	Negative	Right lobe at laparotomy
Case 42	Hepatic cirrhosis	Negative	Negative	Left lobe at laparotomy
Case 43	Hepatic cirrhosis	Negative	Negative	Right lobe at laparotomy
Case 43	Hepatic cirrhosis	Negative	Negative	Left lobe at laparotomy
Case 44	Hepatic cirrhosis	Negative	Negative	
Case 45	Fatty liver	Negative	Negative	
Case 46	Fatty liver	Negative	Negative	
Case 47	Fatty liver	Negative	Negative	
Case 48	Fatty liver	Negative	Negative	
Case 49	Fatty liver	Negative	Negative	
Case 50	Fatty liver	Negative	Negative	
Case 51	Hemolytic jaundice	Negative	Negative	
Case 52	Amebic hepatitis	Negative	Negative	
Case 53	No liver disease	Negative	Negative	
Case 54	No liver disease	Negative	Negative	
Case 55	No liver disease	Negative	Negative	
Case 56	No liver disease	Negative	Negative	
Case 57	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 57	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 58	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 58	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 59	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 59	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 60	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 60	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 61	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 61	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 62	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 62	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 63	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 63	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 64	No liver disease	Negative	Negative	
Case 65	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 65	No liver disease	Negative	Negative	Left lobe at laparotomy
Case 66	No liver disease	Negative	Negative	Right lobe at laparotomy
Case 66	No liver disease	Negative	Negative	Left lobe at laparotomy

* A Klebsiella-like organism not further identified.
† Not believed to be a contaminant; grown directly from a piece of liver tissue.

tube was shaken manually to distribute the particulate material. Ten milliliters of the inoculated medium were then transferred by pipet to another tube and 1 ml. of this was placed in 10 ml. of Brewer's thioglycollate broth. The initial culture was incubated anaerobically under tensions of ten per cent hydrogen, ten per cent carbon dioxide, and eighty per cent nitrogen in Brewer's anaerobic jars. The 10 ml. transfer and the thioglycollate subculture were incubated aerobically at 37° C. Microscopic examination of stained preparations and subcultures to blood-agar plates were made at forty-eight and ninety-six hours from all three liquid media. The blood-agar plates were made in duplicate with one set incubated aerobically and the other anaerobically under the conditions described above. The blood-agar plates were examined for growth at twenty-four hours and forty-eight hours. Positive cultures were identified by the usual bacteriological procedures.

RESULTS

Eighty-five biopsies of human livers were taken from living patients and handled in the above manner. One biopsy was obtained thirty minutes after death from a patient with advanced liver disease. The results of these studies are summarized in Table I.

It can be seen in Table I that there were three cultures that yielded organisms both aerobically and anaerobically (case 35, case 37, and case 38) in the human material. One of these (case 38)

TABLE II

	Diagnosis	Aerobic	Anaerobic
1. Dog 1	Normal liver	Negative	Clostridia
2. Dog 2	Normal liver	Negative	Clostridia
3. Dog 3	Normal liver	Negative	Negative
4. Dog 4	Normal liver	Negative	Negative
5. Dog 5	Normal liver	Negative	Negative
6. Dog 6	Normal liver	Negative	Negative
7. Dog 7	Normal liver	Negative	Negative
8. Dog 8	Normal liver	Negative	Negative
9. Dog 9	Normal liver	Negative	Negative
10. Dog 10	Normal liver	Negative	Negative
11. Dog 11	Normal liver	Negative	Negative
12. Dog 12	Normal liver	Negative	Negative
13. Dog 13	Normal liver	Negative	Clostridia
14. Dog 14	Normal liver	Negative	Clostridia
15. Dog 15	Normal liver	Negative	Clostridia
16. Dog 16	Normal liver	Negative	Negative
17. Dog 17	Normal liver	Negative	Clostridia
18. Dog 3a	Normal liver	Negative	Negative
19. Dog 4a	Normal liver	Negative	Clostridia
20. Dog 5a	Normal liver	Negative	Negative

was in the patient from whom a biopsy was taken thirty minutes after death. There were found growing from this liver a *Streptococcus pyogenes, Escherichia coli, Proteus vulgaris,* and *Proteus mirabilis.* From a specimen of case 35 obtained from the left lobe of the liver at the time of laparotomy there was grown a gram-negative facultative aerobic organism, species unknown, which resembled closely a *Klebsiella.* Also from the left lobe of the liver at laparotomy a fungus was found to grow in case 37 which was of the *Aspergillus* species and believed to be non-pathogenic. This appeared to be growing out of a tiny fleck of liver tissue and was not believed to be a contaminant. None of these patients was receiving antibiotics at the time of biopsy. The cultures from all of the other cases were negative, both under aerobic and anaerobic conditions. The biopsies taken from the dogs, on the other hand, showed a growth in seven of the twenty specimens (Table II). This is considered to be a significant check against both the technic used for obtaining the biopsy specimen and for the bacteriological method of isolation.

DISCUSSION

There is abundant evidence in the literature that not only the liver but other organs of dogs, cats, guinea pigs, hogs and rabbits may harbor bacteria during life (10, 11, 20). The liver has been cited as the source of bacteria or bacterial products resulting in the death of dogs where liver fragments have been placed in the peritoneal cavity (12, 14, 21, 22) Although there has been some controversy as to the actual cause of death in these experiments, it appears likely that it results from the breakdown products of bacterial growth in the liver implanted in the peritoneal space rather than directly from the bacteria themselves (23–26). It has been shown that fresh sterile liver implanted in the peritoneal cavity will not cause death, but fresh sterile liver incubated with added bacteria will cause death when implanted in the peritoneum of a recipient animal (14). The importance of these experimental findings from a practical point of view seem to relate to hepatic trauma either through an external force or secondary to abdominal surgery where hepatic tissue may be left free in the peritoneal cavity (25, 27). Such a possibility also exists when a portion of the liver is rendered relatively anoxic by interruption of its arterial blood supply (17). An actual or potential bacterial flora of the liver may likewise be of some importance in shock where an insufficient oxygenation of the liver may lead to a rapid multiplication of anaerobic organisms.

The role of the liver as a screening organ for bacteria coming from the intestinal tract has received some attention in the past. Berg, Zau, and Jobling (13) pointed out a possible bacteriostatic function of the liver by demonstrating positive cultures from the livers of dogs with negative cultures from pure bile in the same animals. When the bile was diluted, however, these cultures then became positive, suggesting that the bacteriostatic property was altered with dilution. Whipple and Harris (28) have reported four cases of hepatic cirrhosis in whom there was found a *Bacillus coli* septicemia. The authors believed that this reflected an interference of the function of the liver as a bacterial filter due to the circulatory changes of cirrhosis and to a possible lowering of resistance to bacteria. Beeson, Brannon, and Warren (29) conclusively demonstrated in patients with subacute bacterial endocarditis that the liver served as a filtering organ for bacteria. They showed that the concentration of bacteria in the hepatic vein blood was strikingly less than that in the peripheral circulation.

It has not as yet been definitely established that there is seeding of the liver with bacteria coming from the intestine. This seems likely, however,

since it has been shown by Ellis and Dragstedt (14) that fetal dog livers will not produce the "liver autolysis" previously described. It has also been shown by Brim (30) that only six of one hundred livers from human stillborn infants showed positive growth upon culture. In these six cases, the organisms grown were characteristically those present in the intestinal tract. These experiments tend to show that when there are no bacteria in the intestine, as in the fetus, bacteria are not likely to be present in the liver.

That the portal blood contains bacteria has likewise not been established. Arnold demonstrated, on the other hand, by cannulating the thoracic duct in dogs that bacteria could be found in the thoracic lymph five to thirty minutes after they had been placed in the duodenum along with alkaline solutions of egg white (31). Thus, it is entirely possible that such bacteria as are present may have reached the liver and other tissues of animals via the thoracic duct and the general circulation.

The experimental data herein reported show that in hepatic diseases and in normal livers viable bacteria are present in the liver in life only in a very small proportion of cases. As soon as thirty minutes after death, however, multiple organisms may be demonstrated.

Some criticism may be directed at the technic employed in these cases. Since a Vim-Silverman needle was used in all cases, the biopsy undoubtedly came from the peripheral portion of the liver. Trusler and Martin (32) have shown that the stromal elements, that is, blood vessels, bile ducts and connective tissue are far more effective in the production of fatal hepatic peritonitis in dogs than are the parenchymal elements. These authors state that the periphery of the liver contains a relatively large proportion of parenchymal tissue and hence fewer bacteria. It was for this reason that the livers of the dogs used in this study were biopsied and cultured with a technic identical to that used in the human cases. With the same procedure, therefore, dog livers were found to harbor bacteria in a far greater percentage than did human livers.

Of some interest is the fact that growth was demonstrated in the left lobe of two of the cases (case 35 and case 37). The right lobe in case 35 had previously been biopsied and found to contain no bacteria. Trusler and Reeves (33) found that the bacterial content in the left lobe of the liver in dogs is more profuse than in the right lobe. This may be related to the stream lining of portal blood to the liver, causing a greater concentration of organisms or otherwise creating local conditions which are more favorable for bacterial survival and multiplication (34, 35). It should be remembered at the same time, however, that both of these biopsies were taken *at surgery*, and the manipulation of the gastrointestinal tract, the anesthesia administered, or the presence of anoxia may have influenced the finding of bacteria in the liver. The biopsies taken at surgery from the right and left lobes of other cases with hepatic cirrhosis (cases 34, 41, 42, and 43), on the other hand, and from patients with no hepatic disease (cases 57, 58, 59, 60, 61, 62, 65, and 66) showed no growth.

Although it would appear from this study that bacteria ordinarily are not found in the liver of humans, it does not seem possible to conclude that the presence of bacteria in the liver arising from the intestinal tract play no major role in disease. It has been shown that bacteria may invade the liver within a very short time after death. It is also possible that this may take place in certain abnormal physiologic states such as in connection with abdominal trauma, surgery, irradiation, shock, anoxia, or other circumstance leading to local changes in the intestinal wall. These data seem to show, however, that bacteria are much less commonly present in the human liver than in the liver of dogs. Due caution must therefore be exercised in the application to man of experimental data from dogs involving the flora of the liver.

SUMMARY AND CONCLUSIONS

Animal experiments point to a possible role played by the intestinal flora in hepatic disease. The presence of bacteria in the liver suggests an explanation for death due to "liver autolysis" and for death following hepatic artery ligation in dogs. The presence of bacteria in human liver was sought for by means of culture of liver biopsy material under aerobic and anaerobic conditions. Only two of eighty-five cultures from living patients were found to be positive. It may be of some significance that these cultures were taken from the left

lobe of the liver at the time of laparotomy. As a check of the technic, biopsies were taken from dogs and handled in an identical fashion. Thirty-five per cent of these were positive. It is conceded that although under special circumstances bacteria from the intestinal tract invade the human liver and may contribute to morbidity, caution should be exercised in the transfer to man of conclusions drawn from animal experiments.

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ABNORMAL GLYCINE METABOLISM IN RHEUMATOID ARTHRITIS

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Connective tissue is considered to be composed of tightly folded polypeptide chains, resting in an extracellular amorphous ground substance consisting of polymerized muco-polysaccharides. As a result of the pioneering investigations of Meyer (1) and Duran-Reynals (2), considerable investigation has been carried out concerning alterations in composition and permeability of the ground substance in diseases affecting connective tissues (3). Alterations in the polypeptide chains responsible for the tensile strength and elasticity of connective tissue, however, have not yet been adequately studied in these diseases, nor is there any information available concerning the factors responsible for maintaining the integrity of this widely prevalent extracellular protein network. Methods suitable for clinical investigation have been lacking for investigation of this problem. We wish to report an adaptation of the hippuric acid liver function test devised by Quick (4), which has revealed in patients with active rheumatoid arthritis abnormal metabolism of at least one of the major amino acid components of collagen. These patients fail to maintain their serum glycine within 15 per cent of the fasting value after the administration of 1.77 gm. sodium benzoate intravenously.

The basis for our observations rests upon the unique chemical composition of the proteins, collagen and elastin. Glycine constitutes 22.8 to 27.6 per cent by weight of these substances, accounting for approximately one-third of the total amino acid residues in these proteins (5). The glycine content of these proteins is unlike that of any other human extracellular (see Table I) or intracellular proteins whose composition has been described (6). Any widespread increase in collagen synthesis in the body might therefore be reflected in a considerable increase in the requirements of this amino acid. Although glycine is not an essential amino acid, its rate of synthesis is so limited that dietary supplementation is necessary in birds where large amounts of collagen are needed for feather development (7). Furthermore, excessive excretion of glycine as hippurate has been shown to limit body growth in rats (8).

During fasting, the normal serum glycine level of 2.0 to 3.0 mg. per cent is chiefly maintained by diffusion of "readily available" glycine stored in extra-hepatic tissues, since the liver is continuously de-aminating amino acids in circulation. Glycine may also be synthesized in the liver, along with other non-essential amino acids (9). About two-thirds of the glycine conjugated with benzoic acid in the liver, in the hippuric acid excretion test devised by Quick, comes from endogenous sources (10). Serine, sarcosine, and glycolic acid have been shown to contribute to the formation of glycine (11–15). Since a marked reduction in the rate of hippurate formation may occur in some cases of acute or advanced rheumatoid arthritis (16–19), we became interested in learning whether this represented merely reduced liver function, or decreased supplies of "readily available" glycine in peripheral tissues, which prevented the conjugating activity of the liver from acting at full capacity. Estimation of the concentration of serum glycine during the rapid hepatic formation and renal excretion of glycine-benzoic acid conjugates (hippuric acid) was therefore carried out to determine the relative availability of free glycine in cellular reservoirs, and in extracellular

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TABLE I

Glycine and alanine content of various proteins

	Glycine gm. per 100 gm. prot.	Alanine gm. per 100 gm. prot.
A. Intracellular proteins		
1. Muscle	2–5	4
2. Liver	8.5	4.7
3. Human hemoglobin	4.2	9.5
B. Extracellular proteins		
1. Collagen	22.8–23.1	8.1
2. Elastin	24–27.6	0–6
3. Human serum albumin	1.6	
4. Human serum globulin	5	2
5. Fibrin	5.2–5.4	4
6. Bence-Jones protein	2	5
7. Keratin (human hair)	4.5	1.5
8. Insulin	4.4–4.6	2.7–4.5
9. Human casein	0	2.1
10. Human lactalbumin	0	2.7

Values selected from Block and Bolling, VII, pp. 358–361 (6).
(Omission of decimal fractions indicates approximate nature of reported values.)

depots such as collagen and elastin. Simultaneous estimation of serum alanine was carried out as a control, since this amino acid has an entirely different frequency distribution in tissue proteins, and does not directly participate in the detoxification of benzoic acid (Table I). Alanine synthesis is very readily performed in man since glutamic and pyruvic acid contribute to its formation.

METHODS

Most of the patients selected for study were ambulatory, gainfully employed and in nutritional balance. In most cases, several years had elapsed since any previous hospitalization. Serum was obtained from fasting patients for bilirubin determination (*20*), bromsulphalein retention 45 minutes after injection of 5 mg./Kg. (*21*), alkaline phosphatase (*22*), cholesterol ester partition (*23, 24*), total serum protein and A/G (*25*), thymol turbidity (*26*), cephalin flocculation (*27*), and for amino acid determination. In addition to routine urinalysis, fractional phenolsulfonephthalein excretion was carried out on most patients, as a check on renal function.

The modified hippuric acid test of Quick was carried out as follows: After a 12-hour fast, and after the patient had emptied his bladder, an initial venous blood sample was obtained, and 1.77 gm. of sodium benzoate was injected intravenously within a 5-minute period. All urine was collected as separate specimens for the first and second hours after injection, and a second blood sample was obtained at one hour and sometimes at longer intervals. Precautions were taken to ensure an adequate urine flow, usually by ingestion of 200 to 400 cc. of water prior to injection, or a cup of black coffee. Serum glycine and alanine were determined on aliquots of identical serum specimens. The methods of Alexander were used for glycine and alanine estimation (*28, 29*), and those of Weichselbaum were used for determination of hippuric acid excretion (*30*).

The arthritic cases have been classified in Stages I to IV according to the classification of Steinbrocker as adopted by the New York Rheumatism Association (*31*).

RESULTS

At present, 54 patients with rheumatoid arthritis in all degrees of activity and stages of disease have been studied, as well as 41 control patients, the majority of whom had hypertrophic osteo-arthritis, post-traumatic arthritis, gout or metastatic cancer (Tables II, III). Thirty-six of the 41 control patients revealed serum glycine levels which either did not change after sodium benzoate administration, or declined less than 15 per cent at the end of one hour after injection

TABLE II

Initial response of serum glycine to benzoate administration in various clinical conditions

Diagnosis	No. of cases with 15 per cent or greater reduction from initial level	No. of cases with less than 15 per cent reduction from initial level
A. Control patients		
Decompensated hepatic cirrhosis	1	
Metastatic adenocarcinoma	2	5
Traumatic arthritis	1	3
Hypertrophic osteo-arthritis	1	12
Gouty arthritis		1
Psychogenic rheumatism and psychoneurosis		3
Miscellaneous non-arthritic problems such as spondylolysis, calcified bursa, healed spinal fusion, etc.		5
Bronchial asthma		2
Postoperative hemorrhoidectomy		1
Lobar pneumonia, convalescent		1
Chronic dermatitis		2
Meniere's syndrome		1
Total of control patients	5	36
B. Rheumatoid arthritis, peripheral joints		
Stage I	10	8
Stage II	5	3
Stage III–IV	2	1
C. Rheumatoid spondylitis		
Stage I	3	0
Stage II	13	4
Stage III–IV	2	3
Total of rheumatoid patients, including both active and inactive phases of disease	35	19*
D. Rheumatic heart disease		
Active rheumatic fever recurrence	1	2
Inactive	0	2
E. Other diseases involving collagen		
Reiter's syndrome, acute	1	0
Chronic discoid lupus erythematosus	0	1
Spontaneous postoperative wound dehiscence and cortical ovarian fibrosis (Chiari-Frommel syndrome)	1	0

* Nine of these 19 patients on subsequent tests showed a reduction in serum glycine of more than 15 per cent.

TABLE III

Hippurate excretion and serum glycine response in arthritic and non-arthritic patients

Diagnosis	No. pts.	No. obs.	Mean first hour hippurate (expressed as gm. benzoic acid)	Mean per cent change in serum glycine	Standard error of difference between means of control and test series of glycine observations	*t* value of significance*
A. Control patients without bone or joint disease	16	17	0.760±0.282 S.E. 0.071	−5.1±6.7 S.E. 1.7		
B. Control patients with radiologic evidence of joint pathology other than rheumatoid arthritis	18	19	0.789±0.215 S.E. 0.049	−9.3±7.2 S.E. 1.7		
C. Control patients with radiologic evidence of osseous pathology other than rheumatoid arthritis (6 met. carcinomas, 1 Paget's disease)	7	9	0.715±0.340	−7.5±11.7		
Total of controls	41	45	0.764±0.272 S.E. 0.041	−7.4±8.4 S.E. 1.2		
D. Patients with Stage I rheumatoid arthritis (without radiologic signs of disease except soft tissue swelling)	21	34	0.913±0.334 S.E. 0.058	−18.9±14.8 S.E. 2.5	2.8%	4.07
E. Patients with Stage II rheumatoid arthritis (with radiologic evidence of joint pathology but no deformity)	25	46	0.852±0.276 S.E. 0.041	−15.4±13.1 S.E. 1.9	2.3%	3.47
F. Patients with Stage III–IV rheumatoid arthritis (with joint deformity and/or total disability)	8	36	0.778±0.308 S.E. 0.056	−15.7±14.9 S.E. 2.5	2.8%	2.99
G. Miscellaneous patients with other diseases of collagen	8	8	0.775±0.238	−13.4±11.9	4.4%	1.36

* When *t* equals or exceeds 3.0, differences in mean values attributable to chance alone would occur less than once in 370 sets of observations.

(Figure 1). A decline of serum glycine exceeding 15 per cent of the initial fasting level was present in five of the control patients, nearly all of whom had clinical disease of one type or another. Two patients had proven metastatic carcinoma; one was in remission following radiation and testosterone therapy. A third patient also developed a positive test with the progression of hepatic metastases of mammary adenocarcinoma. One patient suffered from decompensated portal cirrhosis with ascites, and another had post-traumatic arthritis of the acetabulum secondary to malposition resulting from an old femoral neck fracture. One patient had roentgenologic evidence of hypertrophic osteoarthritis of the spine, with a persistently normal sedimentation rate. Patients recovering from extensive infections or surgical procedures were among the few control patients whose reduction in serum glycine following benzoate administration approached but did not exceed 15 per cent.

Thirty-five of the unselected 54 patients with rheumatoid involvement showed, on initial examination, a decline of serum glycine greater than 15 per cent of the fasting value at one hour, similar to that seen in Figure 1. Twenty-eight of 29 of

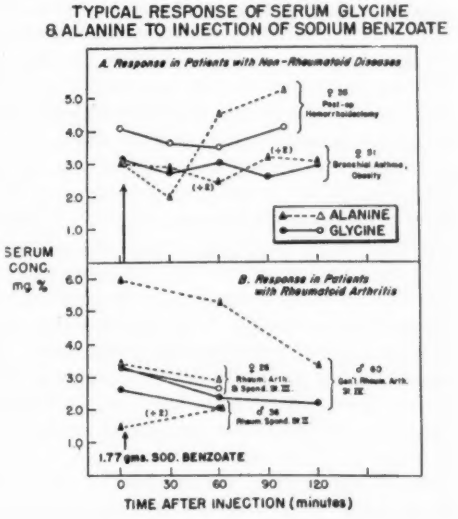
TYPICAL RESPONSE OF SERUM GLYCINE & ALANINE TO INJECTION OF SODIUM BENZOATE
A. Response in Patients with Non-Rheumatoid Diseases
♀ 36 Post-op Hemorrhoidectomy
♀ 21 Bronchial Asthma, Obesity
ALANINE
GLYCINE
SERUM CONC. mg %
B. Response in Patients with Rheumatoid Arthritis
♀ 28 Rheum. Arth. & Spond. St. III
♂ 60 Gen'l Rheum. Arth. St. IV
♂ 36 Rheum. Spond. St. II
1.77 gms. SOD. BENZOATE
TIME AFTER INJECTION (minutes)

FIG. 1

these patients examined on more than one occasion showed at some time a "positive" glycine response falling more than 15 per cent to levels, in some cases, less than 50 per cent of the initial values. When the total series of observations is classified according to the presence or absence of bone or joint involvement in both control patients and in rheumatoid arthritis cases, it is apparent that serum glycine lability is slightly increased in the presence of non-rheumatoid bone or joint pathology (Table III). The changes, however, are far greater in the rheumatoid patients who, in every stage of the disease, show highly significant increases in serum glycine response. This table also illustrates the normal hippuric acid excretion throughout all groups of patients observed; indeed, the Stage I rheumatoid cases show a barely significant increase in mean hippuric acid excretion compared with that of the entire control series. The t value for this increase was 2.36, corresponding to less than 1 chance in 50 of random occurrence. Statistical evaluation of the differences in glycine response indicates that they would occur by chance in less than 1 out of 100,000 trials. No significant differences in alanine levels were noted in either of these two groups of patients (32).

No significant abnormalities of liver function were detectable by laboratory examination in most patients in either the rheumatoid or control series. Infrequently, reduction in hippurate excretion, abnormal thymol turbidity, cephalin flocculation or inversion of the A/G ratio was noted in patients with Stage IV arthritis. Renal insufficiency was not present in any of the cases in the series.

Among the rheumatoid arthritis group, serial determinations of serum glycine response to sodium benzoate administration and of sedimentation rates were frequently carried out. Fifteen patients were observed two or more times with an interval of two or more months intervening between blood samplings. In 11 patients, the sedimentation rate varied more than 10 mm. per hour, and its changes correlated directly with the variation in glycine response to benzoate administration. An increase or decrease in the former was accompanied by a greater or lesser fall, respectively, in the level of serum glycine. In 3 patients, there was no detectable correlation, perhaps because there was less than 10 mm. change in sedimentation rates in the interval. The last patient, Stage IV arthritic, showed wide fluctuations of serum glycine response independent of his sedimentation rate.

DISCUSSION

These observations reveal that patients with active rheumatoid arthritis cannot maintain a constant serum glycine level under the load imposed by intravenous administration of 1.77 gm. sodium benzoate. The data failed to show any corresponding disturbance in hippuric acid excretion, serum alanine concentration, or in hepatic or renal function. This manifestation of altered glycine metabolism has been noted in many Stage I rheumatoid arthritics previous to the development of deformity or roentgenographic abnormalities of bones and joints. It has been noted almost exclusively in patients in whom there were good clinical grounds for suspecting pathologic changes affecting collagen, including patients with active rheumatic fever and disseminated lupus erythematosus (19). Patients with inactive rheumatoid arthritis, inactive rheumatic fever, and discoid lupus erythematosus usually are able to maintain a serum glycine level within 15 per cent of the fasting level, similar to patients without organic disease, and those with hypertrophic osteo-arthritis only.

The decline of serum glycine from fasting levels noted in these patients might be explained by decreased reservoirs of pre-formed glycine in liver or peripheral tissues, by decreased synthesis of glycine, or by diversion of glycine from extracellular to intracellular pathways of utilization (Figure 2). Alteration in the function of enzyme systems controlling the distribution of bound glycine either in polypeptides or in some other form might contribute to the latter possibility. No differences have been noted in the mean daily glycine intake of rheumatoid and non-rheumatoid patients.

Glycine tolerance tests have failed thus far to establish an increased rate of glycine utilization in rheumatoid arthritis. Abnormally flat tolerance curves have been reported by DeVries and Alexander in one case (33), and by Stillman and associates in preliminary observations (34). In neither instance were the changes in these tolerance tests shown to be specific for glycine, and absent for other amino acids. Several groups of ob-

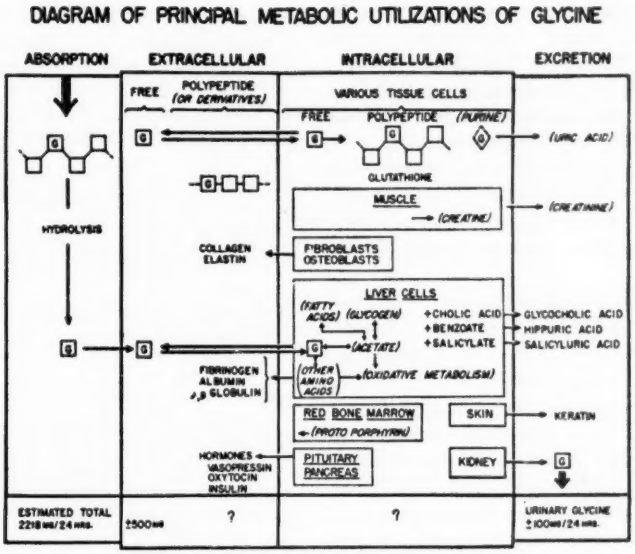

FIG. 2

servers have found no alteration in the fasting levels of glycine in rheumatoid arthritis patients compared to normals (32–34).

Hippurate synthesis is carried on at a normal rate in the Stage II to III arthritics, in spite of the decline of serum glycine. This suggests that the hepatic supply of glycine for detoxification is much the same in rheumatoid as non-rheumatoid subjects, since variations in glycine supply have a marked effect on the rate of hippurate synthesis (35, 36). Therefore, glycine deficiency in these patients, if present, is more likely to have taken place in extrahepatic amino acid depots, such as muscle, bone marrow, or epithelial tissues, if these findings are to be explained.

Many of the alternative processes utilizing glycine in rheumatoid arthritis appear to be depressed rather than increased (Figure 2). The presence of weight loss, anemia, hypo-albuminemia, muscular weakness and atrophy, cutaneous dystrophy (accompanying psoriatic skin manifestations), and cutaneous atrophy in advanced rheumatoid arthritis speak for reduced purine, hemoglobin, creatine, and keratin synthesis compared to normal. Only the synthesis of collagen appears to be increased in some of these cases, as a part of the reparative processes (37).

Both clinical and laboratory evidence therefore favor an extrahepatic source for the abnormalities noted in glycine metabolism in patients with rheumatoid arthritis and allied conditions. Further investigation is necessary to determine whether there are alterations in enzyme mechanisms releasing it to the blood stream, or using it for protein synthesis.

These observations of altered amino acid metabolism in rheumatoid arthritis have important etiologic and therapeutic implications that cannot be overlooked. Because of the uniquely small size of glycine residues, relative to those derived from other amino acids, the high concentrations of glycine in collagen and elastin have often been thought significant in influencing the characteristic configurations and modes of packing of the polypeptide chains in these fibrous proteins (38, 39). Failure of the supply of glycine to the periarticular fibroblasts would lead to impairment in production of normal collagen, or to the formation of abnormal collagen of poor quality. No analysis of the glycine content of diseased collagen has yet been made.

Excess free glycine administered parenterally or orally increases the rate of hippurate formation by the normal or diseased liver (4, 33, 35, 36). Pa-

tients with severe manifest anxiety-reactions or normal individuals under severe stress excrete unusually large amounts of hippurate (40, 41). Many of our rheumatoid patients showed chronic anxiety, and the early cases in Stage I excreted more hippurate than did controls or those with more advanced disease (Table III). Severe emotional trauma superimposed upon chronic anxiety and insecurity is among the predisposing mental conditions favoring the onset of rheumatoid arthritis (42).

Re-evaluation of nutritional factors would appear to be important in the prevention and treatment of the rheumatoid group of diseases, not only because of these findings, but because of the limited daily intake of and synthetic capacity for glycine compared to its manifold pathways for utilization in essential processes (Figure 2). Although therapy with cortisone and ACTH delays the uptake of amino acids by rat cells (43), we have failed to note in our patients treated with these compounds any immediate improvement in abnormal serum glycine response. Spontaneous disease remissions, however, are often associated with return to a normal response curve.

SUMMARY

The administration of sodium benzoate to patients with active rheumatoid arthritis results in a decline of more than 15 per cent in the fasting level of serum glycine in the majority of cases, which persists for at least one hour. Similar changes have been observed in patients with active rheumatic fever and disseminated lupus erythematosus. Serum glycine remains constant in healthy persons or patients with hypertrophic osteo-arthritis. Because of the uniquely high concentration of glycine in collagen and elastin (22.8 to 27.6 per cent) and the fact that abnormal response of serum glycine to benzoate administration has been noted chiefly in diseases characterized by pathology of collagen, this test may be indicative of a metabolic abnormality affecting connective tissues. Evaluation of the response of serum glycine to benzoate administration may assist in the objective evaluation of patients with arthritic symptoms and in the earlier recognition of rheumatoid arthritis.

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A STUDY OF THE NATURE OF THE CIRCULATING THYROID HORMONE IN EUTHYROID AND HYPERTHYROID SUBJECTS BY USE OF PAPER ELECTROPHORESIS[1]

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The physico-chemical form in which the secretion of the thyroid gland exists in blood is not well understood. It is generally believed to be associated in some manner with the plasma proteins since the iodine content of precipitated washed plasma protein (1) and the rate of turnover of I^{131} in this protein appears to vary with the state of thyroid activity (2). The observation of Trevorrow (3) that this "protein bound" or "hormonal iodine" is readily extractible with butanol from unhydrolysed serum indicates that it is not bound by a peptide linkage.

The introduction of the technique of paper electrophoresis combined with the use of I^{131} as a tracer isotope has afforded a new and valuable instrument for a study of the problem (4, 5). With this technique it has been demonstrated that after 24 to 48 hours there is a characteristic localization of administered I^{131} in the alpha globulin moiety although the exact location is still somewhat in doubt (4–6). It is the purpose of this paper to study further this localization and to compare euthyroid and hyperthyroid subjects particularly with regard to the turnover of iodine in these two groups over a period of several days.

[1] This study was supported in part by the Wisconsin Alumni Research Foundation.

[2] Research Fellow of the Arthritis and Rheumatism Foundation.

[3] The radioactive iodine used in this study was obtained from the Isotopes Division of the Oak Ridge National Laboratory of the Atomic Energy Commission, Oak Ridge, Tenn.

CLINICAL MATERIAL

The six patients selected for this study were seen in the Department of Medicine of the University of Wisconsin. Thyroid function was measured by determination of serum protein bound iodine by the method of Barker (7), and by the 24-hour uptake of I^{131},[3] as reported previously (8). Pertinent clinical data are summarized in Table I. Three patients were hyperthyroid as judged by their classical symptoms, physical findings, elevated PBI, and I^{131} uptake. Three patients were euthyroid as judged by similar criteria, but were given radioactive iodine therapy for incapacitating cardiac disease. In each instance therapeutic quantities of carrier-free I^{131} ranging from 5 to 23 mc. were administered orally. These doses were calculated to deliver the same amount of radiation (75 to 100 μc/gm.) to the gland in all subjects. This estimation was based upon previous uptake studies of "tracer" quantities of I^{131} (100 μc.) and upon the size of the gland.

Upon completion of the diagnostic studies and administration of the radioactive iodine, blood samples were drawn at daily intervals, placed in dry centrifuge tubes, and the serum separated for paper electrophoresis.

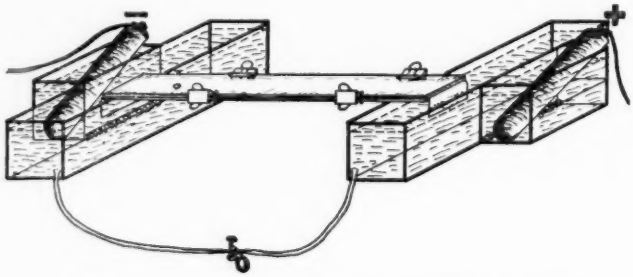

FIG. 1. SKETCH OF THE PAPER ELECTROPHORESIS APPARATUS
Described in text.

TABLE I

Summary of clinical data

Patient	Age	Sex	Diagnosis	Physical findings	Protein bound iodine*	I^{131} Uptake† at 24 hrs.	I^{131} Administered
					μgm. %		mc.
1. (C. S.)	19	F	Hyperthyroidism	Diffuse goiter. Estimated wt. 75 gm.	11.1	58%	8
2. (M. V.)	47	F	Hyperthyroidism	Diffuse thyroid, barely palpable. Estimated wt. 30 gm.	11.3	51%	5
3. (J. W.)	45	M	Hyperthyroidism	Diffuse goiter. Estimated wt. 60 gm.	10.2	61%	7
4. (N. R.)	57	M	Euthyroid Hypertensive, arteriosclerotic heart disease Old myocardial infarct Angina decubitus	Thyroid not palpably enlarged. Estimated wt. 25 gm.	5.0	15%	16
5. (E. S.)	50	F	Euthyroid Hypertensive heart disease Paroxysmal auricular fibrillation	Diffuse thyroid, barely palpable. Estimated wt. 30 gm.	7.3	38%	9
6. (V. D.)	34	F	Euthyroid Luetic heart disease Aortic insufficiency Cardiac enlargement Massive anasarca	Thyroid not palpably enlarged. Estimated wt. 25 gm.	4.8	15%	23

* Normal range PBI 4 to 8 μgm.%.

† I^{131} uptake expressed as per cent of the dose present in the thyroid at 24 hours. (Euthyroid range, 10–40%; hyperthyroid, over 50%.)

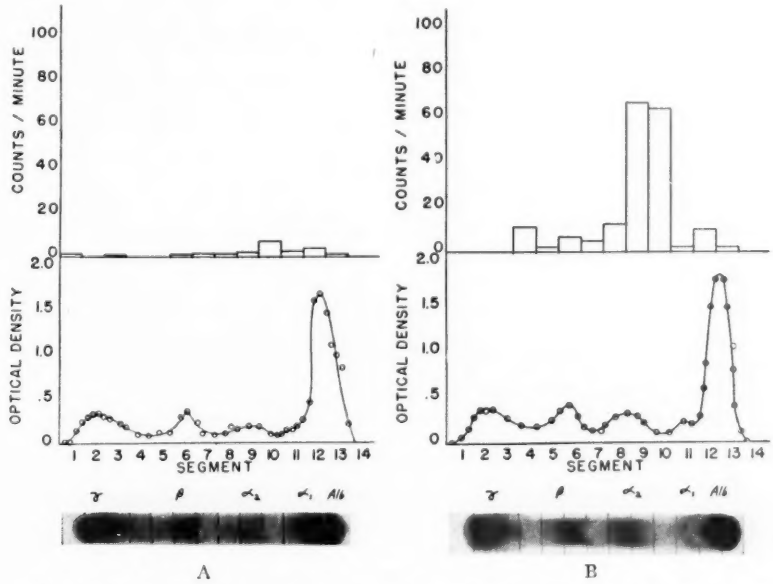

FIG. 2. LOCALIZATION OF PROTEIN-BOUND I^{131} BY PAPER ELECTROPHORESIS IN A EUTHYROID (A) AND A HYPERTHYROID (B) PATIENT, EIGHT AND TWO DAYS RESPECTIVELY, AFTER I^{131} ADMINISTRATION

The bottom strips are photographs of the stained paper strips. The middle graphs represent the protein concentration of the strips (dye elution method). The top graphs show the relative radioactivity of the segments.

METHODS

1. *Filter paper electrophoresis.* The method of Kunkel and Tiselius (9) was employed with a few modifications. A strip (3.5 × 45 cm.) of Schleicher and Schuell No. 598 filter paper was soaked in 0.05 M veronal buffer, pH 8.6, and clamped between two glass plates (3.8 × 35 cm.) after rolling the paper heavily with a photographic roller in order to remove excess buffer and air spaces. The glass plates were first covered with a thin coat of silicone grease and the sides of the plates were sealed together with the grease after the filter paper was clamped in place. The plates were then placed across a pair of leucite buffer vessels, each containing 1 liter of the veronal buffer with the free ends of the paper dipping into the buffer (Figure 1). A period of one hour was allowed for equilibration of the buffer on the paper. The equilibration and actual electrophoresis were done in a cold room at 2° C. Carbon electrodes were introduced into separate vessels filled with buffer, the latter being connected to the

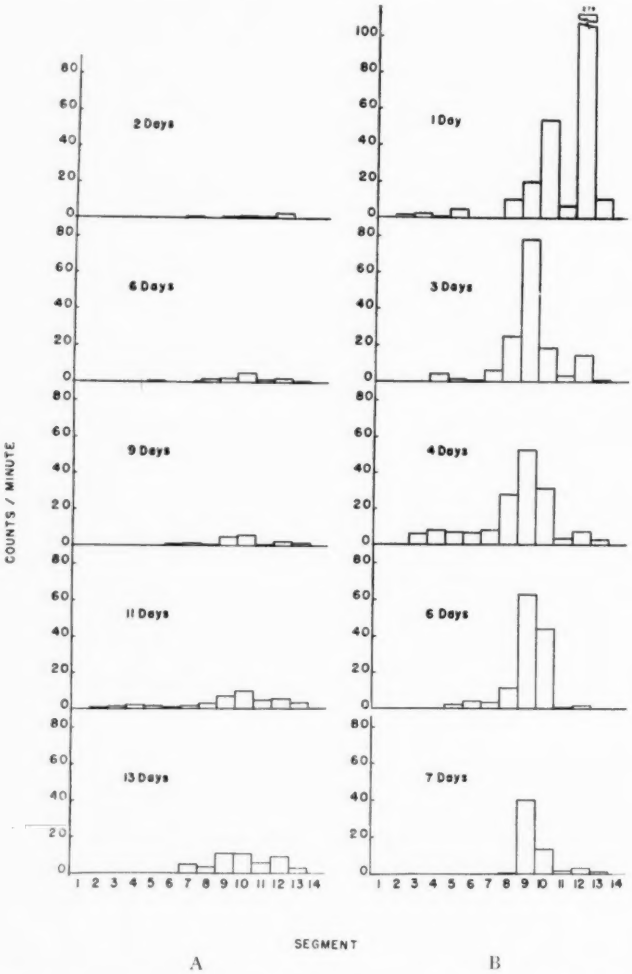

FIG. 3. COMPARISON OF SERIAL RADIOELECTROPHORETOGRAMS IN A EUTHYROID (A) AND A HYPERTHYROID (B) PATIENT

The radioactivity was normalized for dose of I^{131} and corrected for isotopic decay.

main buffer vessels through a slot filled with glass wool to prevent pH changes during the operation. Fifty lambda of serum were introduced to the paper strips through a small hole in the upper glass plate 9 cm. from the cathodal end and allowed to drain evenly into the paper. After 30 minutes a potential of 300 volts D.C. was placed across the electrodes. With this system approximately 1 milliampere will pass through each paper strip. Twenty hours were allowed for resolution at this current and the strips were then removed.

2. *Counting technique.* At the completion of the electrophoresis the strip was dried in an oven at 80° C. The dried strip was then divided transversely into fifteen 2 cm. segments, using for purposes of consistent orientation the starting point of the electrophoresis. The first segment was cut exactly 5 cm. toward the cathodal end from this point, thus placing the starting point of electrophoresis in the center of segment three. The radioactivity of each segment was determined with a mica window bell-type Geiger counter using a slotted leucite sample holder which assured reproducible positioning. A minimum of 500 counts per segment was recorded.

3. *Quantitative estimation of protein.* After the segments were counted they were subdivided into 0.5 cm. sections and the protein bands identified and quantitated by the bromphenol blue elution method (9).

RESULTS

Figure 2 represents the radioelectrophoretograms of representative euthyroid and hyperthyroid patients. The radioactivity is located in two major zones, which are identical in both subjects. One zone is centered in the slowest moving portion of the albumin (segment 12), but distinctly ahead of the alpha-1 fraction. The other is just ahead of the alpha-2 fraction in an area of relatively low protein concentration (segments 9 and 10). This distribution of radioactivity was observed in all the patients studied.

Although the radioactivity is confined to the same two zones in both the euthyroid and hyperthyroid subjects there is a distinct difference in the absolute and relative concentration at these two sites in these two groups. This is particularly striking when serial radioelectrophoretograms done at daily intervals are compared (Figure 3). In these two series the radioactivity measurements were normalized (standardized to correct for differences in the quantity of I^{131} administered) and corrected for decay. The total radioactivity is much greater in the hyperthyroid than in the euthyroid subject throughout the series. Initially, most of the radioactivity is associated with the albumin fraction in both the euthyroid and hyperthyroid subjects. However, the radioactivity at this site in the hyperthyroid subject declines rapidly. In the euthyroid subject, on the other hand, although the initial albumin-bound radioactivity was small, it increases progressively. With the rapid decline of radioactivity bound to albumin in the hyperthyroid subject, there is a rapid reciprocal rise in radioactivity at the alpha globulin zone (segments 9 and 10). The activity at this alpha globulin zone in the euthyroid subject is initially low and increases gradually. Figure 4 compares the turnover of I^{131} at the alpha globulin zone in three euthyroid and three hyperthyroid subjects.

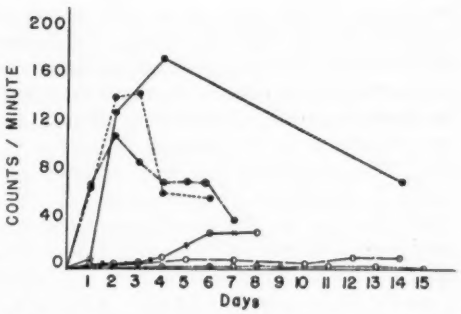

FIG. 4. RELATIONSHIP OF RADIOACTIVITY IN ALPHA GLOBULIN (SEGMENTS 9 AND 10) TO THE TIME INTERVAL AFTER ADMINISTRATION OF I^{131} IN THREE HYPERTHYROID (SOLID DOTS) AND THREE EUTHYROID (OPEN DOTS) SUBJECTS

The radioactivity was normalized for dose of I^{131} and corrected for isotopic decay.

DISCUSSION

During the first few hours after the administration of I^{131}, most of the radioactivity rapidly passes off the paper strip toward the anode (4, 5). It is assumed that this fraction is free iodide ion since this is the behavior which would be expected of a small negatively charged ion. Serial radioelectrophoretograms demonstrate that the radioactivity remaining on the strip, and hence presumably "protein-bound," increases progressively so that at the end of the three days all but a small fraction remains on the strip (5).

The concentration of I^{131} in the albumin fraction soon after its administration is believed to represent partial binding of the iodide by this protein, whose ion-binding capacity is well known. This

assumption is supported by *in vitro* studies in which Na I^{131} was added to serum. A radioelectrophoretogram showed significant localization of radioactivity in the albumin fraction even though the greater part moved faster than albumin and was assumed to be the unbound radioactive "I" ion. This observation has been reported previously by Gordon and his associates (4). The observation that the albumin of hyperthyroid subjects bound and released I^{131} at more rapid rates than the albumin of euthyroid subjects may reflect a more rapid turnover of total body iodine in hyperthyroid subjects.

In both euthyroid and hyperthyroid subjects the major portion of the albumin bound iodine is associated with the slowest moving portion of albumin suggesting a difference in iodine binding capacity within the albumin fraction. This differential iodine binding within the albumin fraction is consistent with Salter's observation (10) that the greater part of the PBI was in Cohn's fraction VI which contains the smaller albumin molecules.

The rapid shift of I^{131} to the protein fraction having electrophoretic mobility slightly faster than alpha-2 globulin (sections 9 and 10) in the hyperthyroid patients and the slower but significant I^{131} concentration in the same area in euthyroid subjects, suggests that the circulating thyroid hormone is, or is associated with, this alpha globulin fraction. The unequivocal localization of iodine to this fraction by paper electrophoresis is in contrast to earlier reports (11) indicating that hormonal iodine was associated with albumin. Most of this investigation was done using ammonium sulphate protein fractionation. It is now known that much of the alpha globulin is precipitated with albumin by this method. Analysis of Cohn's globulin fractions, however, has indicated a high iodine-to-protein ratio in fraction IV–2 which contains largely alpha globulins (10).

Present evidence indicates that the biologically active iodine fraction in blood is a thyroxine-like substance (3, 12) complexed with a plasma protein. *In vitro* experiments (4) demonstrate that radio-thyroxine added to plasma becomes bound to an alpha globulin, supporting the view that the circulating thyroid hormone may be a thyroxine-alpha globulin complex.

It is generally believed that patients with hyperthyroidism have a quantitative rather than a qualitative difference in the circulating iodine compounds (13, 14). Chromatographic analysis of iodine-containing compounds in protein fractions separated by paper electrophoresis should give valuable additional information.

The possibility that accumulation of radioiodine in the alpha-2 area is due to artefact attendant upon radiation damage to the thyroid gland has been considered. The demonstration by Robbins and his associates (15) that a rise in serum PBI and an increase in the ratio PBI/butanol-extractible iodine after administration of large doses of I^{131}, might seem to support this possibility. We believe this unlikely for the following reasons: *1*) All subjects both euthyroid and hyperthyroid received the same concentration of I^{131}/gm. tissue; *2*) Robbins and his associates report no change in the PBI/butanol extractible iodine ratio in their subjects who received doses of I^{131} comparable to those given in the present report; *3*) When radiothyroxine is added to serum *in vitro* it appears in the alpha globulin area (4).

SUMMARY AND CONCLUSIONS

1. The sera of three euthyroid and three hyperthyroid subjects have been studied using I^{131} as a tracer isotope and paper electrophoresis for separating the major plasma protein components.

2. After twenty-four hours the radioactivity localized in two major zones, one in the slowest moving albumin and the other in a zone of low protein concentration just ahead of alpha-2 globulin. This localization was seen in both euthyroid and hyperthyroid subjects.

3. Soon after administration of I^{131} most of the radioactivity was associated with the albumin fraction but in the hyperthyroid individual there was a rapid shift to the alpha region. A similar though considerably slower shift occurred in the euthyroid subjects.

4. Present evidence indicates that the hormonal iodine is carried by the alpha globulin fraction. The tracer iodine concentrated faster, attained higher levels, and declined more rapidly at this site in the hyperthyroid than in the euthyroid subjects.

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MUSCLE COMPOSITION IN RESPIRATORY ACIDOSIS [1]

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The close relationship of muscle composition to the electrolyte pattern of extracellular fluid was described by Darrow (1) and confirmed by others (2). The direct correlation of serum bicarbonate concentration with the intracellular sodium content of muscle in metabolic acidosis and alkalosis was demonstrated by these workers. Until the present time it has not been determined whether this relation of serum bicarbonate to muscle sodium applies in respiratory disturbances of acid-base balance. In these states serum pH varies inversely with serum bicarbonate concentration or serum pCO_2, in contrast to metabolic disturbances in which serum pH varies directly with serum bicarbonate concentration and serum pCO_2. Thus, by the study of tissue composition in respiratory disturbances, it can be established whether muscle composition is related under all circumstances to serum pH, serum bicarbonate concentration or serum pCO_2.

This paper presents analyses of serum and muscle of rats in whom respiratory acidosis was induced by inhalation of gas mixtures containing 10 to 15 per cent carbon dioxide for periods of fourteen to twenty-one days.

PLAN OF EXPERIMENTS AND METHODS OF ANALYSIS

Sixteen male albino rats weighing 300 to 400 gm. were kept in an air-tight lucite chamber into which was run a mixture of compressed air and carbon dioxide. Group A, eight animals, was exposed to an atmosphere containing 11 per cent CO_2 for two weeks. Group B, eight animals, was exposed to 13 per cent CO_2 for three weeks. Analysis of the air in the chamber was performed at frequent intervals throughout the experiments by means of the Roughton Scholander apparatus (3). The oxygen concentration in the chamber did not fall below 17 per cent.

The temperature within the chamber was maintained at 23° C. and the relative humidity at 80 per cent. The chamber was opened for twenty minutes every three days for the purpose of cleaning the cages and replenishing food [4] and distilled water.

All animals were anesthetized with ether within one to three minutes after removal from the chamber, and blood was obtained anaerobically from the dorsal aorta immediately thereafter.

The methods used in the analysis of serum and muscle are the same as described earlier by this laboratory (4) except that sodium and potassium were determined by a Barclay internal standard flame photometer. Serum pH was determined on the Beckman pH meter using a temperature correction of 0.0118 pH units for each degree of temperature below 38° C. The pH of the sera so obtained was not considered representative of the pH of the animals while in the chamber, since the high alveolar carbon dioxide tension would be rapidly dissipated during anesthesia. The values for pH, bicarbonate and carbonic acid concentrations of serum while in the chamber cannot be determined exactly and were calculated as indicated below.

The average percentage of deviation from the mean of duplicate determinations was as follows:

$$Cl \pm 1.0\% \quad P \pm 1.1\% \quad Na \pm 1.1\% \quad K \pm 1.2\%$$

Calculations

Group A—

$$ppCO_2 \text{ chamber} = 0.11 \times (760 - 47) = 78,$$

$$pCO_2 \text{ blood}[5] = 85 \text{ mm. Hg approximately.}$$

Group B—

$$ppCO_2 \text{ chamber} = 0.13 \times (760 - 47) = 93,$$

$$pCO_2 \text{ blood}[5] = 100 \text{ mm. Hg approximately.}$$

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[2] John and Mary R. Markle Scholar in Medical Science.

[3] This study represents a portion of the thesis submitted for the degree of Doctor of Medicine, Yale University School of Medicine.

[4] Purina Fox Chow:—Na—34 mM./100 gm.; K—13 mM./100 gm.; Cl—17 mM./100gm.; N—2.95 gm./100 gm.; P—36 mM./100 gm.

[5] The percentage of carbon dioxide in the alveolar air must be greater than that in the chamber. Normally, CO_2 tension in the lungs is about 40 mm. Hg greater than in the air. However, Brown, Hemingway, and Visscher (6) demonstrated a progressive decrease in this gradient

TABLE I

Body composition in disturbances of acid-base balance

Group	Reference	Serum						Muscle per 100 gm. FFS					
		Na mM./L.	K mM./L.	Cl mM./L.	HCO_3 mM./L.	pH	pCO_2 mm. Hg	H_2O gm.	Cl mM.	Na mM.	K mM.	P mM.	Nai mM.
Normal	1,2,4,8	141–146	4.1–5.4	99–102	21–25	7.35	45	334–346	5.1–7.2	9.4–10.6	46.1–48.9	32.0–34.0	3.1
Respiratory acidosis													
A	—	136–147 (141)*	4.3–5.3 (5.1)	90–96 (93)	31–37 (32)	7.25†	85	312–335 (326 ±7)	4.7–5.8 (5.0 ±0.4)	8.4–9.7 (9.0 ±0.4)	47–49 (48 ±1)	32–34 (33 ±1)	3.3–3.8 (3.5 ±0.2)
B	—	142–144 (143)	4.5–5.0 (4.8)	82–87 (85)	37–46 (40)	7.24†	100	320–359 (333 ±12)	4.8–5.7 (5.3 ±0.4)	7.3–9.8 (8.8 ±0.9)	47–50 (48 ±1)	32–35 (33 ±1)	1.1–3.3 (2.2 ±0.8)
Metabolic alkalosis	8	151	3.4	87	35	7.44	53	314	3.9	11.9	37.8	30.4	7.7
Metabolic acidosis	8	142	6.7	94	20	7.28	44	337	4.5	6.1	49.8	33.2	1.6
Respiratory alkalosis (altitude)	9	143	5.6	124	↓	↑	↓	342	9.1	12.0	45.0	32.5	3.5
H. R.	—	135	5.2	80	46	—	—	366	13.0	20.0	42.4	—	1.8
Normal human muscle	7							360–400	12–15	16–22	41–44	—	2–6

* Mean.
† Calculated from carbon dioxide content and probable pCO_2 of alveolar air while in chamber.

pH_{ser} [6] of animals while in the chamber was calculated from the Henderson-Hasselbach equation using pCO_2 above and carbon dioxide content obtained by analysis plus 5 mM. per liter for the change in cation-binding capacity of blood proteins with change in pH as described by Singer and Hastings (5). The rapid loss of dissolved CO_2 which occurred during exsanguination produced marked change in the serum pH, but would have relatively less effect on the serum carbon dioxide content.

$$[H_2CO_3]_{ser} \text{ in mM./L.} = pCO_2 \times .0301,$$

$$[HCO_3]_{ser} \text{ in mM./L.} = CO_2 \text{ content} - [H_2CO_3]_{ser}.$$

Electrolyte concentration of extracellular fluid as mM./Kg. of water was calculated by the method described by Yannet and Darrow (4).

$$\frac{[Cl]_{ser}}{[H_2O]_{ser} \times 0.95} = [Cl]_e,$$

$$\frac{[Na]_{ser} \times 0.95}{[H_2O]_{ser}} = [Na]_e,$$

$$[K]_e = [K]_{ser}.$$

Muscle per 100 gram fat-free solids (FFS)

$$(H_2O)_e \text{ in grams} = \frac{(Cl - 1)}{[Cl]_e},$$

$$(Na)_e \text{ in mM.} = (H_2O)_e \times [Na]_e,$$

$$(Na)_i \text{ in mM.} = (Na) - (Na)_e.$$

RESULTS

All animals tolerated the experiment well with little overall change in weight in either group. Hyperpnea was evident early in the experiment, but activity was not obviously diminished. Analysis of serum in both groups revealed a marked rise in serum carbon dioxide content and fall in serum chloride concentration from the normal (Table I)—changes characteristic of chronic respiratory acidosis. The mean total serum carbon dioxide content in Group A was 35 ± 2 mM./L. and in Group B, which was exposed to 12 to 15 per cent CO_2 for three weeks, it was 43 ± 3 mM./L. Since the carbon dioxide concentration in the alveolar air must have been higher than the concentration in the chamber, the pCO_2 of arterial blood was at least 85 mm. Hg in Group A and 100 mm. Hg in Group B. These values correspond to carbonic acid concentrations of 2.6 mM./L. and 3.0 mM./L. respectively. Thus the mean serum bicarbonate concentration by calculation was 32 ± 2 mM./L. in Group A and 40 ± 3 mM./L. in Group B.

The mean serum pH of animals in Group A

as the environmental CO_2 pressure increased. The assumption that the alveolar pressure of CO_2 in this experiment is 7 mm. Hg higher than that of the atmosphere would seem to be a reasonable one.

[6] Subscripts "ser," "e," and "i" refer to serum, extracellular, and intracellular phases, respectively.

( ) refers to total amount in mM. or gm.

[ ] refers to concentration.

while in the chamber was calculated to be 7.25, whereas Group B averaged 7.24.

The serum chloride concentration was low in each group, 93 ± 2 mM./L. and 85 ± 2 mM./L. in Groups A and B, respectively.

The serum sodium and potassium concentrations were not significantly altered in either group, although the mean potassium concentration was at the upper limit of normal.

The analysis of skeletal muscle from these animals revealed relatively little change from normal composition (Table I). The changes in Group B were somewhat greater than in Group A. The sodium content was 8.8 ± 0.9 mM./100 gm. FFS in Group B. This value was slightly lower than the normal range (9.4–10.6 mM./100 gm. FFS) obtained by Darrow (1). The muscle potassium (48 ± 1 mM./100 gm. FFS) was at the upper limit of the normal range (46.1 – 48.9 mM./100 gm. FFS). The chloride and phosphorous content of muscle was within normal limits (5.3 ± 0.4 mM./100 gm. FFS and 33 ± 1 mM./100 gm. FFS, respectively).

The derived values for intracellular sodium $(Na)_i$ of muscle were somewhat lower than normal. The mean $(Na)_i$ for Group B was 2.2 ± 0.8 mM./100 gm. FFS in contrast to the normal value of 3.1 mM./100 gm. FFS given by Darrow (1).

The water content of muscle was not significantly altered but the extracellular phase may have been decreased in Group A.

Analysis of human muscle (H. R.—Table I) obtained at postmortem examination from a patient with chronic respiratory acidosis on a basis of cystic fibrosis of the lungs and pancreas revealed findings similar to those described above in rats. Table I also shows the composition of normal human muscle (7). Human muscle potassium is lower than that of rats, probably 40 to 44 mM. per 100 gm. of fat-free solids. The analyses are shown to demonstrate that marked changes in intracellular Na and K did not occur in one patient dying with extremely high total CO_2 due to compensated respiratory acidosis.

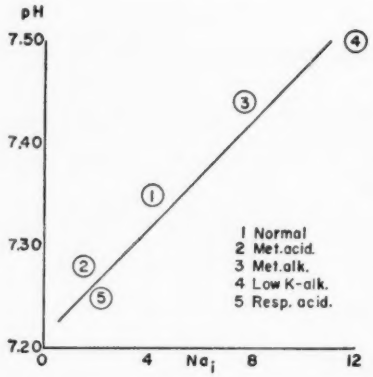

FIG. 1. RELATION OF INTRACELLULAR SODIUM OF MUSCLE IN MM./100 GM. FFS TO PH OF EXTRACELLULAR FLUID

References and values from Table I.

DISCUSSION

The results of this study indicate that muscle composition is not especially altered in chronic respiratory acidosis, despite the presence of low serum chloride concentration and high serum bicarbonate concentration in this condition. The changes in muscle composition which do occur are in the same direction as those noted in metabolic acidosis (Table I) rather than metabolic alkalosis.

If the values for serum bicarbonate and intracellular sodium in respiratory acidosis are plotted on the graph formulated by Darrow and his associates (1) for animals with metabolic acidosis, metabolic alkalosis, and potassium deficiency alkalosis, there is wide variance from the regression line previously calculated.

Similarly, if $(Na)_i$ of muscle is plotted against the pCO_2 of serum as calculated from the data of Cotlove and his associates (8) and the present data, no correlation is noted.

However, in Figure 1, serum pH in all these disturbances, respiratory and metabolic, is plotted against $(Na)_i$. It is obvious that there is a direct relation between intracellular sodium and extracellular pH in all these states. Likewise muscle potassium varies inversely with serum pH.

These findings are consistent with the data of Darrow and Sarason (9) who demonstrated slightly high intracellular muscle sodium and somewhat low muscle potassium in rats exposed to low atmospheric pressures. These animals undoubtedly had some degree of respiratory alkalosis secondary to overbreathing (10), since serum so-

dium was normal and serum chloride was elevated (Table I). Unfortunately serum carbon dioxide content and serum pH were not obtained.

Thus, it is likely that the loss or gain of potassium or sodium by the cells is dependent not upon extracellular bicarbonate or chloride concentration or carbon dioxide tension, but rather upon the pH of the extracellular fluid. In general the following predictions may be made:

1. That in chronic states, in which extracellular pH is elevated, muscle potassium is lowered and intracellular sodium elevated.
2. That in chronic states, in which extracellular pH is lowered, muscle potassium is normal or elevated and intracellular sodium reduced.
3. That the changes in muscle composition are considerably less in respiratory disturbances of acid-base balance than in metabolic disturbances.

According to the hypothesis of Wallace and Hastings (11) intracellular pH is dependent upon PCO_2 of body fluids since intracellular bicarbonate concentration is constant. According to this concept intracellular acidosis is present in metabolic alkalosis with respiratory compensation, as well as in respiratory acidosis, since pCO_2 is elevated in both conditions. However, the composition of muscle was strikingly different in the two types of disturbances. This fact suggests the following alternatives:

1. That electrolyte composition of muscle *is not* related to intracellular pH if the hypothesis of Wallace and Hastings is valid, or
2. That electrolyte composition of muscle *is* related to intracellular pH and the hypothesis of Wallace and Hastings that intracellular pH is directly related to pCO_2 of body fluids is not valid.

Which proposition is correct cannot be determined at the present time. The data of Conway (12) suggest that a large part of the total acid labile CO_2 in muscle measured by Wallace and Hastings may be barium-soluble and hence not bicarbonate. Under these circumstances the pH within the cell would be 6.0 and small changes in intracellular bicarbonate could not be determined by the methods now available.

The finding of relatively normal muscle composition in respiratory disturbances suggests that cell transfer plays a minor role in the correction of such conditions and that renal compensation is of primary importance. These findings confirm the suspicions of Elkinton, Squires, and Singer in the case of humans with respiratory acidosis (13). Therefore, in the fluid therapy of these states adequate quantities of water and extracellular electrolyte (sodium chloride) should be provided so that complete renal compensation may be effected.

SUMMARY

Male rats were exposed to an atmosphere containing 10 to 15 per cent carbon dioxide for two to three weeks. Analysis of serum at the end of that time revealed changes characteristic of chronic respiratory acidosis, namely high serum bicarbonate concentration (41 mM./L.) and low serum chloride (85 mM./L.). Analysis of muscle revealed potassium content at the upper limit of normal, 48 mM./100 gm. fat-free solids (FFS) and the intracellular sodium was somewhat reduced, 2.2 mM./100 gm. FFS. These findings are in striking contrast to those in metabolic alkalosis in which serum bicarbonate concentration is also high and serum chloride low.

It is suggested that muscle composition is related to the pH of the extracellular fluid and not to the extracellular bicarbonate concentration or carbon dioxide tension.

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STUDIES IN INTRAVASCULAR COAGULATION. I. COAGULATION CHANGES IN ISOLATED VENOUS SEGMENTS [1]

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Significant advances have recently been made in our understanding of the biochemistry of blood coagulation. Most of this work has, of necessity, been performed on blood *in vitro*. *In vivo* studies have been hampered by the difficulty of producing clots and thromboses in animals under controlled conditions and by the fact that, unlike the hemorrhagic diseases, there are no known clinical states in which a specific defect responsible for hypercoagulability of the blood has been demonstrated.

We had been impressed by the repeated references in the literature to the effect that a stationary column of blood in a vein included between two carefully applied ligatures will remain liquid for weeks (1, 2). The experiments referred to were carried out more than seventy-five years ago and review of the original data did not indicate clearly whether or not clotting had occurred in these segments (3, 4). It occurred to us that a study of what *did* happen to the clotting mechanism in isolated endothelial-lined segments might provide a bridge between our knowledge of test tube coagulation and of the clotting process as it evolves in a more physiologic environment. Some of the results obtained are herewith presented.

Clot Formation in the Isolated Segment

Healthy dogs of both sexes, 9 to 47 Kg. in weight, were anesthetized with intraperitoneal nembutal, the neck and groin were shaved and cleansed with soap and water and an intravenous infusion of normal saline was begun. Aseptic precautions were not attempted. Both femoral and external jugular veins were widely exposed: the former from Pouparts ligament to within 2 centimeters of the knee; and the latter from the base of the neck to the angle of the jaw. All demonstrable tributaries to these veins were carefully ligated with No. 3 zero Deknatel silk and then cut so that the vessels were entirely freed from surrounding structures. Continuity of each vein with the remainder of the circulation was interrupted by the careful application of a 27 mm. seraffine [2] clamp at each end of the freed segment. In larger animals, the isolated femoral veins could be separated into two, and the cervical veins into three segments, each containing approximately 1 cc. of blood. After isolation, the segments were covered with gauze moistened with saline, and the wound closed by apposition of the skin edges with a hemostat. This procedure required one to two hours. Hemostasis was well maintained; rectal temperature, pulse rate, and hematocrit remained constant throughout the operation.

At selected intervals vein segments were removed from the animal and by release of one clamp the contents were emptied into clear shallow glass dishes containing 2 to 3 cc. of 2.5 per cent sodium citrate. These dishes were carefully inspected for the presence or absence of a gross clot. The presence of clot, regardless of size, was taken as the end-point. The empty vein segments were always opened and examined for residual clots.

In fourteen dogs the time interval was observed between the isolation of the vein segment and the recognition of the clot in thirty-four segments. Clot formation was never observed in less than twenty minutes, usually not until after thirty minutes had elapsed and in a few segments not until more than one hour had passed. When these clots were examined promptly by phase microscopy they were found to consist principally of fibrin. These initial clots were very small, but increased only slowly with time. That coagulation was not complete for at least as long as eight hours after isolation could be demonstrated by emptying the contents of such segments into containers devoid

[1] This work was carried out under a contract between the office of the Surgeon General, U. S. Army, and the President and Fellows of Harvard College.

[2] Manufactured by Arthur H. Thomas, Philadelphia, Pa.

of anticoagulant in which further clot formation could be observed.

Other Alterations in the Blood in the Isolated Segment

The obvious retardation of coagulation afforded by endothelial tissue was further pursued by investigating other clotting functions in these isolated segments; namely—prothrombin consumption, accelerator formation and platelet disappearance. In another group of twelve dogs similarly prepared, the blood was emptied into siliconized centrifuge tubes containing 0.5 cc. of 3.2 per cent sodium citrate. Under these conditions gross clots were not evident unless the blood was obtained from segments which had been isolated for two or more hours.[3]

[3] Small clots, visible in twenty to thirty minutes when blood was diluted with large amounts of anticoagulant in shallow containers as described above, were not recognizable in these centrifuge tubes where blood dilution was minimal.

One-stage prothrombin values were obtained by the modification of Rosenfield and Tuft (5) in which pooled barium sulfated dog plasma was used as the diluent. Two-stage prothrombin determinations were obtained by the method of Ware and Seegers (6) except that the blood was not defibrinated prior to measurement. Platelets were counted by the direct method with Rees-Ecker diluting fluid (7). Control 1 cc. blood samples carefully obtained from superficial veins with Arquad [4] coated needles and siliconized syringes, were transferred promptly to siliconized centrifuge tubes containing 0.5 cc. of 3.2 per cent citrate. This volume and concentration of anticoagulant was found to give consistent prothrombin values with 0.5 to 4 cc. of blood. Because of the small amounts of blood available for analysis and the relatively large volumes of anticoagulant in the collecting tubes, the hematocrit was routinely determined to correct for the initial dilution.

[4] A non-wetting agent generously provided by Armour Laboratories, Chicago, Ill.

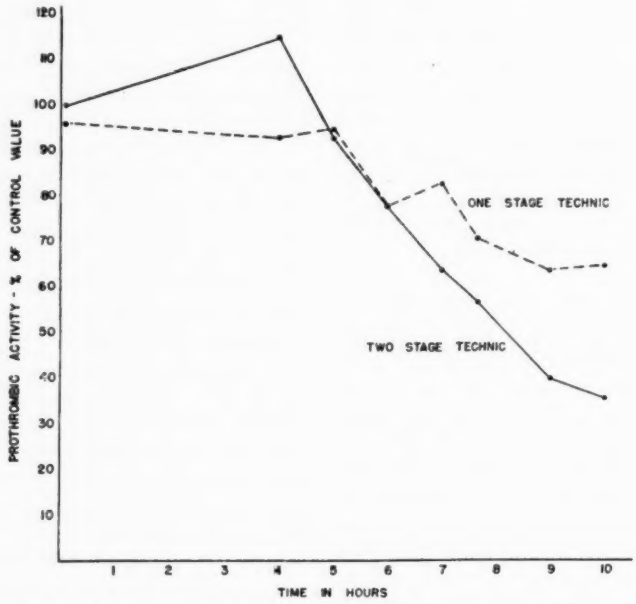

FIG. 1. ONE- AND TWO-STAGE PROTHROMBIN VALUES DETERMINED IN PARALLEL ON THE SAME ANIMAL AT INTERVALS OVER A TEN-HOUR PERIOD

Grossly visible clots appeared sometime before the fourth hour.

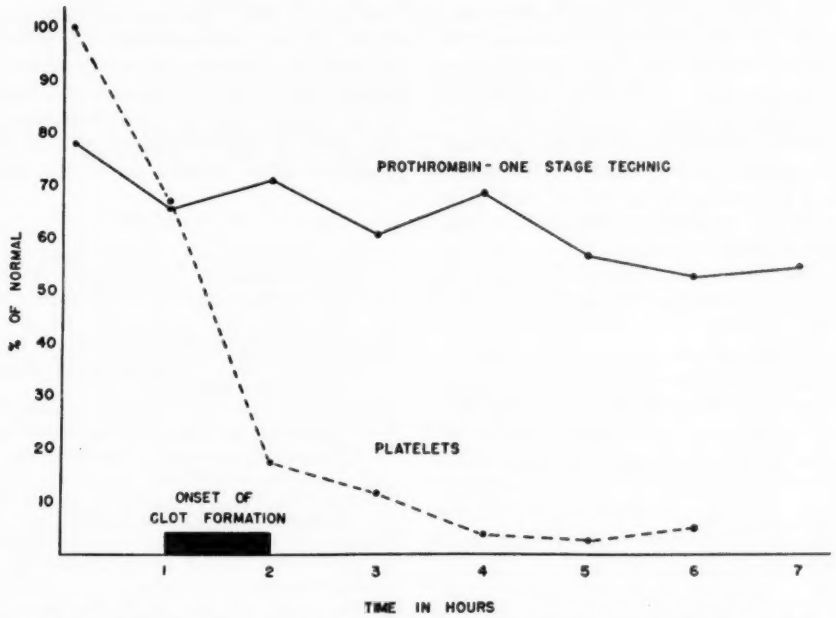

FIG. 2. PLATELET COUNTS AND ONE-STAGE PROTHROMBIN VALUES DETERMINED IN PARALLEL ON THE SAME ANIMAL AT INTERVALS OVER A SEVEN-HOUR PERIOD

Grossly visible clots appeared sometime between the first and second hour.

In a series of 70 segments from 12 dogs, no significant changes in one- or two-stage prothrombic activity were noted until after a segment had been isolated for four hours. Thereafter, the activity decreased although in some instances the change was not apparent until after seven hours. Even after ten hours of isolation a considerable amount of prothrombic activity was still demonstrable. This is consistent with the finding that clots after hours in blood-filled isolated segments are small and increase in size when the blood is subsequently emptied into uncoated glass vessels. The decrement in prothrombic activity as measured by the two-stage method was always greater than that found with the one-stage technic.

In Figure 1 are shown the one- and two-stage values determined in parallel on the same animal at intervals over a ten-hour period; grossly visible clots appeared sometime before the fourth hour—several hours before any demonstrable change occurred in prothrombic activity. Figure 2 represents determinations, over a seven-hour period, of the platelet counts and one-stage prothrombin values in an animal in which clot formation began before the second hour. Although platelet disappearance is delayed markedly over that found when blood is allowed to clot in glass tubes, a correlation can be seen between the onset of platelet disappearance and the development of a grossly visible clot. Again, the one-stage prothrombin values were unchanged throughout the period during which samples were collected.

DISCUSSION

Although prolongation of both the clotting (8) and prothrombin (9) times of blood in silicone tubes have been demonstrated, it is evident that coagulation occurs far more slowly in blood in contact with the endothelial-lined surfaces of isolated venous segments than in coated vessels currently employed in various *in vitro* technics. This is true even though the blood is completely stagnant and the vascular segment cut off entirely from the circulation.

The interpretation of the observed discrepancy between the one and two-stage prothrombin values inevitably depends upon one's viewpoint regarding the specificity of these two measures of the prothrombin moiety. The observed difference is entirely consistent with the view that during the coagulation process changes occur, whereby there is an increase in the velocity with which prothrombin is converted to thrombin. Since this velocity effect is manifest only in the one-stage method (10), the difference between the one- and two-stage values reflects the presence of accelerators.

Of particular interest in this study was the insight afforded into the early phases of the coagulation sequence. From these experiments it is apparent, for instance, that a fibrin clot can develop prior to the disappearance of demonstrable amounts of prothrombin or the elaboration of measurable quantities of clot accelerators. This observation is of potential clinical significance since induced hypoprothrombinemia is currently employed to inhibit or retard intravascular coagulation in man. It becomes important, therefore, to ascertain whether induced hypoprothrombinemia actually causes retarded fibrin deposition *in vivo* under the conditions of these experiments. Work in this direction is in progress.

SUMMARY

The influence of the isolated venous segment of the dog on intravascular coagulation has been characterized with respect to clot formation, prothrombin consumption, accelerator formation and platelet disappearance. Under the conditions of these experiments it has been shown that a fibrin clot may be deposited intravascularly prior to the demonstrable utilization of prothrombin. The isolated venous segment provides an additional and more physiologic means for investigating the early phases of intravascular coagulation, because endothelial tissue replaces artificial membranes.

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URINARY EXCRETION OF AMINO ACIDS IN PATIENTS WITH CIRRHOSIS OF THE LIVER AND IN NORMAL ADULTS [1, 2]

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Although the liver occupies an important position in protein and amino acid metabolism, abnormalities in the metabolism of these nutrients by patients with severe hepatic diseases have been difficult to define. Alterations in the excretion of some amino acids, however, have been reported in patients with liver disease (1, 2). That these patients deviate from the normal with regard to the metabolism of certain individual amino acids also has been observed. For example, the finding of tyrosine and leucine crystals in the urine is considered an indication of severe liver disease, especially "acute yellow atrophy" (3), and abnormalities in the utilization and excretion of administered tyrosine (4, 5), methionine (6), and glycine (7) have been reported.

This study presents data concerning the urinary excretion of the eight amino acids essential for adult man (8) and of arginine and histidine in 20 patients with cirrhosis of the liver as compared to 14 normal individuals maintained on diets and other conditions of study which were comparable.

MATERIALS AND METHODS

The twenty patients studied had advanced cirrhosis of the liver, associated in most instances with chronic alcoholism and decreased food intake. Evidence for severe liver disease was derived from the clinical history, physical examination, and liver function tests as well as liver biopsy or necropsy examination in some patients (Table I). The duration of the liver disease varied considerably. Some patients followed a course of slow improvement, and others chosen for special studies outlined below improved more promptly. The normal subjects were house officers and medical students in good health.

The patients were studied on the Thorndike Metabolic Ward where complete urine collection and dietary control were maintained. The daily food intake provided from 2,000 to 3,200 calories, and was considered adequate in other dietary essentials (except protein in some cases as indicated below). No supplements of methionine, choline, or vitamins were given to these patients during the studies (except patient F. C. who received a multivitamin capsule daily).

Three methods of study were employed. First, the daily urinary excretion of the ten "essential" amino acids was determined in 20 patients with cirrhosis of the liver and in 14 normal individuals maintained on comparable diets. Second, the effect of an increase in dietary protein intake upon the urinary excretion of these amino acids was observed in four of the patients (F. C., R. H., A. C., J. M.). During these studies the patients were given constant daily diets, and in successive metabolic periods of from six to nine days, increases in protein intake were made. In these cases the pooled urine obtained during the last three days of each study period was used for amino acid analysis in order to exclude the period of adjustment due to the change in the protein content of the diet. Finally, the effect of the severity of the liver disease upon the urinary excretion of the ten amino acids was noted in three patients (M. L., M. S., and J. S.). These patients were given con-

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[2] A preliminary report of this investigation appeared in the Proceedings of the National Meeting of the American Federation for Clinical Research, Atlantic City, New Jersey, May 4, 1952.

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TABLE I

Clinical and laboratory data in patients with cirrhosis of the liver

Patient	Age and sex	Ascites*	Edema*	Liver†	Spleen†	Serum bilirubin, mgm. %		Urine		Serum albumin, gm. %	Thymol		B.S.P. % retention	Biopsy (B) or autopsy (A)
						1 min.	15 min.	Bile*	Urobilinogen		Turb.‡	Flocc.*		
J. M.	56 M	0	±	18	N.P.	0.3	1.1	0	1:64	3.5	5.3	4+	36	
M. L.	38 M	1+	±	10	N.P.	1.2	2.5	1+	1:64	2.4	3.8	4+	28	
A. C.	37 M	0	1+	7	14	1.0	3.3	1+	1:32	2.7	3.9	4+	32	B—Chronic cirrhosis
R. H.	38 M	1+	0	12	N.P.	10.0	20.0	4+	1:32	2.2	4.7	0	—	A—Chronic cirrhosis
F. C.	36 F	0	0	8	N.P.	0.5	0.7	0	1:8	3.0	0.9	0	7	B—Marked fatty infiltration
J. S.	40 M	1+	0	8	N.P.	0.5	1.6	1+	1:64	3.0	5.6	3+	48	
W. C.	70 M	3+	2+	N.P.	N.P.	0.1	0.5	0	1:8	2.6	—	—	25	A—Chronic cirrhosis Hepatoma
R. K.	43 M	2+	1+	6	3	0.3	1.1	0	1:128	2.5	2.7	0	—	
C. M.	50 M	2+	0	2	N.P.	0.4	1.2	0	—	2.5	4.2	3+	—	A—Chronic cirrhosis
J. G.	48 M	3+	1+	7	N.P.	0.5	1.4	1+	1:256	2.0	2.2	0	42	A—Chronic cirrhosis
A. B.	44 M	4+	4+	12	N.P.	3.2	5.7	3+	1:256	2.3	3.4	0	—	
P. S.	47 M	4+	3+	6	N.P.	1.3	3.4	2+	1:128	2.1	3.6	3+	40	
B. B.	46 F	4+	1+	7	4	0.4	0.7	0	1:32	2.3	2.3	3+	25	
M. S.	42 M	3+	2+	15	7	2.1	4.2	3+	1:128	2.0	6.7	3+	—	
E. C.	52 F	3+	0	N.P.	N.P.	9.5	30.7	4+	1:160	—	1.8	0	—	B—Acute yellow atrophy
F. F.	51 M	0	0	6	N.P.	2.3	4.7	2+	1:256	1.8	1.8	2+	—	B—Chronic cirrhosis
R. V.	50 M	2+	2+	5	2	0.5	1.3	0	—	—	—	—	—	
T. G.	34 M	1+	1+	7	3	3.5	6.9	2+	—	2.9	5.5	4+	—	B—Cirrhosis and fatty infiltration
W. C.	53 M	1+	1+	6	N.P.	3.8	7.5	3+	1:256	2.8	3.9	3+	—	
S. P.	32 M	4+	4+	7	N.P.	3.6	8.5	4+	1:100	1.7	1.6	1+	—	

* 0–4+.
† Cm. below costal margin.
‡ Normal value <1.7 cc. $BaSO_4$ suspension.
N.P. Not palpable.

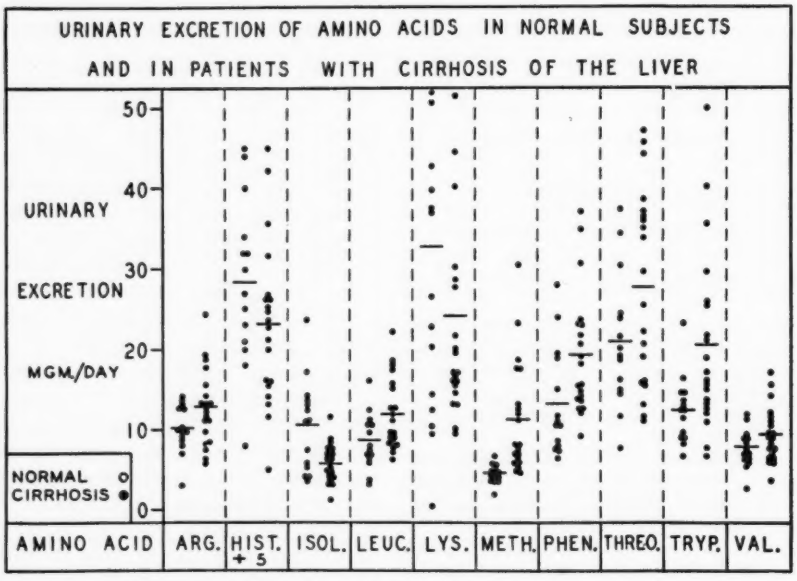

FIG. 1. THE HORIZONTAL LINE IN EACH COLUMN INDICATES THE AVERAGE DAILY URINARY EXCRETION VALUE FOR EACH AMINO ACID

stant daily diets providing at least 2,800 calories, and 100, 75 and 50 gm. of protein per day, respectively, for the nine to 36 days of study. The urines were pooled in three day periods and analyzed for their contents of amino acids for the first three or four pools and also for pools 8 and 12 in the case of J. S. A normal adult (T. P.) was studied in an identical manner for 39 days during which he consumed a constant diet providing 3,000 calories and 75 gm. of protein daily.

The 24 hour urines were preserved with 10 ml. of a 5 to 1 mixture of glacial acetic acid and toluene and refrigeration. Individual amino acid analyses were done by microbiological techniques for the eight amino acids essential for man and for arginine and histidine (9). Plasma and urine alpha amino nitrogen were determined by the gasometric ninhydrin methods described by Van Slyke, McFayden, and Hamilton (10, 11). Laboratory tests of liver functions included serum bilirubin concentration (12), bile in the urine (13) and urobilinogen (14), serum albumin concentration (15), thymol turbidity (16, 17) and flocculation, and retention of intravenously administered bromsulphalein (5 mgm. per kilogram of body weight at 45 minutes) (18).

TABLE II

Urinary excretion of amino acids in patients with cirrhosis of the liver and in normal adults (mgm. day)

	Cirrhosis of liver		Normal adults	
No. subjects	20		14	
	Range	Ave.	Range	Ave.
Dietary protein intake, gm./day	0–125	66	55–125	77
Arginine	5.8–24.4	12.8	3.0–14.0	10.2
Histidine	25–225	116	38–222	142
Isoleucine	1.2–11.6	5.8	3.8–23.6	10.6
Leucine	6.4–22.2	12.0	3.3–16.2	8.7
Lysine	9.7–90.5	24.2	0–100	32.9
Methionine	4.1–30.5	11.4	2.0–6.8	4.6
Phenylalanine	9.2–37.4	19.4	6.6–28.0	13.4
Threonine	11.7–47.1	27.8	7.7–37.5	21.1
Tryptophan	6.9–50.2	20.6	6.8–25.4	12.5
Valine	3.6–17.2	9.5	2.7–11.9	7.8
Total	146–483	259.5	115–430	263.3

RESULTS

1. *Urinary excretion of the 10 "essential" amino acids*

The daily excretion of each of the ten "essential" amino acids in each of the 20 patients with cirrhosis of the liver is compared to that of fourteen normal individuals presented in Table II and in Figure 1. The daily protein intake of these patients varied from 0 (0.2 gm. of nitrogen) to 125 gm. Sixteen of the twenty patients were consuming either 50, 75, or 100 gm. of protein per day, which approximates a normal intake. All patients consuming 50 gm. or more of protein daily were in positive nitrogen balance. The results (Figure 1) show a fairly wide scattering above and below the average values for patients and the normal controls. In most instances this scattering was greater for the patients. The average urinary excretion of each amino acid was slightly greater for the patients than for the normal individuals in the case of seven of the amino acids (arginine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine) but was decreased in three (histidine, isoleucine, and lysine). The overlap between patients and controls, however, was so great that little significance can be attached to differences in the averaged values except in the cases of methionine, of tryptophan, and of isoleucine. In the case of methionine, more than half of the daily determinations in the patients were above the highest value found for the controls, and in some instances was increased five to six times the normal average of 4.6 mgm. daily. The average daily excretion of tryptophan was almost twice that observed in the control subjects, and in six instances was from two

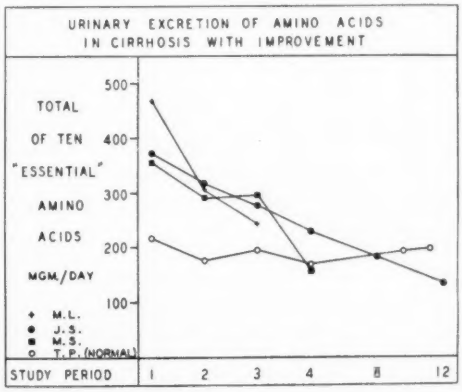

FIG. 2

TABLE III

Effect of dietary protein intake on the urinary excretion of "essential" amino acids (mgm./day) in patients with cirrhosis of the liver and in a normal adult

Patient	F. C.		R. H.		A. C.		J. M.			B. S.* (normal)		
Dietary protein, gm./day	0	100	25	75	100	150	50	75	100	0	75	150
Arginine	14.8	16.4	14.3	13.8	12.9	8.5	10.6	7.7	10.4	7.0	10.1	8.6
Histidine	99	74	79	121	115	156	74	129	237	65	99	140
Isoleucine	4.3	9.2	4.8	4.2	9.2	6.0	7.7	7.1	6.6	13.5	14.4	16.1
Leucine	9.1	13.0	11.2	6.6	18.6	11.6	7.9	11.6	12.0	9.7	11.3	12.9
Lysine	17.2	25.0	20.5	19.0	27.5	12.2	13.7	17.0	19.9	11.8	12.5	11.4
Methionine	3.5	6.7	17.3	8.1	13.4	25.0	5.1	8.2	8.4	2.8	4.0	4.7
Phenylalanine	15.3	13.7	11.2	13.8	28.9	21.9	14.8	24.7	22.0	10.9	15.1	15.9
Threonine	13.9	17.3	25.2	21.8	37.5	28.0	19.6	25.3	29.8	14.8	18.9	20.1
Tryptophan	4.5	11.2	14.6	23.7	19.6	22.4	22.3	31.5	33.6	15.8	23.4	27.4
Valine	13.0	15.4	9.8	7.6	11.1	13.0	10.1	9.0	20.7	9.0	11.9	13.7
Total	194.6	201.9	208.9	239.6	293.7	304.6	185.8	271.1	400.4	160.3	220.9	270.8

* See reference 19.

to four times the normal average of 12.5 mgm. daily. The average daily excretion of isoleucine by the patients was about one-half that noted in the control group, and all but one of the 20 patients with cirrhosis excreted less of this amino acid than the average value found for the normal subjects.

2. Effect of dietary protein intake upon urinary amino acid excretion

The urinary excretion of amino acids was determined in four of the patients during successive periods in which increases in dietary protein intake were made (Table III). The excretions of amino acids in these patients with liver disease are compared with those previously observed in a normal adult maintained on diets of various protein intakes (19). Although alterations in the quantity of dietary protein consumed was accompanied by variations in the urinary excretion of amino acids in the four patients, they were comparable in magnitude to those in the normal individual. Large increases in the dietary protein intake were not accompanied by the urinary wastage of large quantities of these amino acids.

3. Effect of improvement in liver diseases upon urinary excretion of amino acids

The urinary excretion of amino acids was followed during improvement in severe active liver disease in three patients (M. L., J. S., M. S.), Figure 2 and Table IV. The diet of each patient was constant throughout these studies and provided 50, 75, and 100 gm. of protein daily, respectively. Each metabolic period consisted of three days during which the 24 hour urine collections were pooled prior to analysis. Distinct clinical improvement occurred between the successive periods. The excretion of the total of the ten "essential" amino acids is shown in Figure 2 and of the changes in excretion in the individual amino acids in Table IV. The urinary excretion of the total of the ten "essential" amino acids decreased for each of the three patients as improvement occurred. A similar decrease in the urinary excretion of the individual amino acids toward or to normal is demonstrated.

Data obtained on a normal subject (T. P.) maintained on a constant diet providing 75 gm. of protein daily are also included. This subject was studied for a comparable period of time, and the specimens assayed were collected, stored and handled in all respects in a manner identical to those obtained from the patients with liver disease. No decrease in the urinary excretion of the total of the ten essential amino acids or of the individual amino acids was found. The urinary excretion of these amino acids, in fact, remained quite constant during this control study. In addition to pointing out a difference in the urinary excretion of amino acids in the patients with hepatic cirrhosis as compared to the normal individual, the assay results on the "normal" specimens indicate that the decrease observed in the patients is not due to hydrolysis of bound amino acids occurring in the specimens during storage.

TABLE IV

Urinary excretion of "essential" amino acids (mgm./day) in cirrhosis of the liver with improvement

Patient	M. L.			M. S.				J. S.						T. P. (normal)					
Dietary protein, gm./day	100			75				50						75					
Study period	1	2	3	1	2	3	4	1	2	3	4	8	12	1	2	3	4	10	11
Ascites*	1+	±	0	3+	2+	1+	0	1+	1+	±	0	0	0	0					
Liver size, cm.†	11	10	10		17	15	12	9	8	7	5	3	1	0					
Serum bilirubin, mgm. %	3.5	2.7	2.5	4.2	2.4	1.4	0.9	1.9		1.6	1.2	0.5	0.4	0.6					
Thymol: Turbidity‡		3.8	3.7	6.7	5.8	5.5	5.5	4.4		5.6	3.8	2.8	1.8	1.1					
Flocc.*		4+	4+	3+	3+	3+	3+	3+		3+	3+	0	0	0					
B.S.P. retention, %	36	34					23	48					7						
Urine: Bile*	3+	2+	0	3+	2+	TR	0	1+		1+	0	0	0	0					
Urobilinogen	1:128			1:128	1:64	1:64	1:64	1:64		1:64	1:32	1:32	1:32	1:8					
Urine vol., ml./day	1,077	1,600	1,045	3,470	1,950	1,737	1,933	1,343	1,300	970	1,167	898	1,013	2,100	1,927	1,717	1,945	1,963	1,757
Arginine	12.9	12.0	10.7	24.4	25.0	16.8	11.8	13.2	11.6	11.3	9.9	10.1	8.2	17.6	13.5	12.4	10.3	15.2	15.6
Histidine	229	143	106	133	111	141	53.2	192	166	142	111	88	56	126	107	104	97	106	107
Isoleucine	8.9	5.8	6.6	8.8	9.0	6.2	5.6	9.5	7.6	7.5	7.5	5.8	5.3	2.9	4.6	3.6	2.6	3.1	3.9
Leucine	14.5	9.1	10.3	17.7	14.9	12.1	10.9	14.5	11.2	11.0	10.6	8.7	7.8	9.7	10.0	9.4	7.0	8.8	8.4
Lysine	71.3	37.0	23.6	44.6	33.7	29.3	15.3	32.8	24.4	22.9	19.5	12.2	11.5	8.8	9.8	8.9	7.6	8.8	12.3
Methionine	22.2	19.0	16.2	11.4	8.0	6.8	5.6	9.1	7.8	6.9	6.0	5.3	4.8	6.1	6.0	5.8	3.1	6.1	6.1
Phenylalanine	20.1	13.3	12.1	37.4	25.0	15.8	13.7	20.8	17.4	16.6	13.7	9.7	8.0	6.1	6.0	8.6	6.2	8.6	6.0
Threonine	57.4	41.3	32.4	36.2	31.6	33.0	19.1	49.6	42.3	34.8	29.2	22.4	14.8	22.7	24.1	24.6	19.6	23.2	22.7
Tryptophan	18.6	16.2	15.2	25.7	18.7	21.5	14.1	16.7	15.7	13.5	13.3	12.0	10.9	9.2	7.7	8.2	7.0	7.7	8.3
Valine	12.9	10.1	8.2	15.6	14.6	10.9	9.7	12.5	10.8	9.1	9.7	7.5	6.4	6.9	6.9	7.6	5.4	7.1	7.2
Total	467.8	306.8	241.3	354.8	291.5	293.4	159.1	370.7	314.4	275.6	229.3	181.7	133.7	216.0	175.4	193.1	165.8	194.6	197.5

* 0–4+.
† Cm. below costal margin.
‡ Normal value <1.7 cc. $BaSO_4$ suspension.

(All assays were done at the completion of each study, at which time the initial specimen obtained had been stored for several weeks.)

DISCUSSION

The results presented indicate that the urinary excretion of each of the ten "essential" amino acids in patients with cirrhosis of the liver does not differ markedly in quantity from that observed in normal individuals maintained on comparable diets. Likewise, the pattern of the amino acids excreted was comparable for the two groups studied. The values obtained in the patients with liver disease were somewhat more variable than the values in the normal individuals. The average excretion values for seven of the amino acids (arginine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine) were slightly higher in the patients than in the normal subjects, whereas the average excretion of three of the amino acids (histidine, isoleucine, and lysine) were slightly lower in the patient group. An increased urinary excretion of methionine and of tryptophan and a decreased excretion of isoleucine by the patients with liver disease were the most marked deviations from the normal noted. These results confirm and extend the observations concerning the individual amino acid excretion previously reported from this laboratory (1) and by others (2).

It does not seem likely that the increase in urinary amino acid excretion, observed in these patients occurs in large enough quantity to be nutritionally significant or to increase the requirement for these amino acids. In the case of methionine, which is excreted in greatest excess, the figure again does not implicate this amino acid as a limiting one. Moreover, supplements of methionine (and choline) do not enhance the positive nitrogen balance of patients with cirrhosis of the liver (20). The failure to detect gross deviations from the normal urine amino acid excretion in these patients does not imply that the intermediary metabolism of each of these amino acids proceeds normally. In fact, abnormalities in the intermediary metabolism of methionine have been demonstrated in patients with liver disease (6).

The significance of the altered amino acid excretion of these patients is not at once apparent. Elevated plasma concentrations of amino acids presumably do not account for this. In a series of ten patients with severe liver disease, the average plasma alpha amino acid nitrogen concentration was 3.8 mgm. per 100 cc. (range 2.7 to 4.5) as compared to that for 35 normal individuals of 3.7 mgm. per 100 cc. (range 2.9 to 4.5) (Table V). Slight reductions in the renal threshold for amino acids, or a continuously elevated plasma amino acid concentration not great enough to be reflected in determinations of the plasma alpha amino nitrogen concentration, constitute another possible explanation for the aminoaciduria. An elevation of plasma methionine concentration has, for example, been observed (6).

TABLE V

Plasma and urine alpha amino nitrogen in patients with cirrhosis of the liver and in normal adults

	Cirrhotic patients	Normal adults
Plasma: mgm./100 ml.		
No. subjects	10	35
Range	2.7–4.5	2.9–4.5
Average	3.8	3.7
Urine: mgm./day		
No. subjects	15	10
Range	84–258	118–199
Average	171	152

Aminoaciduria occurs in Wilson's disease (hepato-lenticular degeneration) and is much greater in quantity than that of the patients reported here (21, 22). Investigations led to the conclusion that an alteration in renal excretion best accounted for the abnormality (22). The pathological changes in the liver in the patients with Wilson's disease studied were usually much milder than in the patients with cirrhosis of the liver, so that, although it is possible that the aminoaciduria of the two diseases are related, that occurring in the former condition cannot be attributed to accompanying hepatic cirrhosis.

Since the protein intakes of the patients studied were in most instances within the range of that for the normal adults, forced protein feeding was not the cause of the slight increases in urinary amino acid excretion. It was concluded from a previous study of a normal adult that a large proportion of the amino acids excreted in the urine were "endogenous," and not related to food intake (19). The findings in the patients were comparable to those in the normal adult receiving diets of varied protein contents.

A progressive diminution in the urinary excretion of amino acids was observed in some patients during improvement in their disease as evidenced clinically and by laboratory tests of liver function. In some instances urinary excretion values which were distinctly elevated returned to well within the normal range. This phenomenon, which has also been observed by others (*2*), may explain, in part at least, the greater variation noted in the amino acid excretion of the patients with liver disease than of the normal subjects, since this is influenced more by the severity and stage of the patient's disease than by the dietary protein intake. The nutritional implication of these observations is not apparent, but suggests a more efficient utilization of amino acids with improved liver function. Whether the progressive decrease in amino acid excretion noted is unique in patients with liver disease or also occurs in patients recovering from various other illnesses has not been determined.

Gross abnormalities in the urinary excretion of "essential" amino acids by patients with cirrhosis of the liver were not found in the present study or in previous studies. The metabolism of the so-called "non-essential" amino acids, however, has received little attention in these patients. It is conceivable that any limitations of protein synthesis occurring in patients with liver disease may be attributable to a limited ability of such patients to synthesize amino acids. Therefore, the possibility exists that an amino acid which is "non-essential" for normal man may be "essential" for the patient with hepatic disease in order to insure maximal body protein synthesis and repletion in this disease state.

SUMMARY AND CONCLUSIONS

The urinary excretion of the eight amino acids essential for adult man and of arginine and histidine were determined microbiologically in 20 patients with cirrhosis of the liver and in 14 normal individuals maintained on comparable conditions of study. In selected metabolic studies the effect of increases in dietary protein intake, and in other studies the effect of the severity of the liver disease upon the urinary excretion of these amino acids, was determined.

The results of these studies indicate:

1. The urinary excretion of the eight amino acids essential for man and of arginine and histidine does not differ markedly in quantity, or in pattern, in the patients with cirrhosis of the liver when compared to normal individuals.

2. The average excretion values for arginine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine were slightly higher in the patient group than in the control group, and those for histidine, isoleucine, and lysine were slightly lower.

3. An increased urinary excretion of methionine and of tryptophan, and a decreased excretion of isoleucine by the patients were the most marked deviations from the normal.

4. Large increases in the quantity of protein consumed by four of the patients did not result in the urinary wastage of amino acids.

5. Improvement in liver disease in three patients was accompanied by progressive decreases in the urinary excretion of these amino acids.

6. It is concluded that alterations in the urinary excretion of amino acids by patients with cirrhosis of the liver depend more upon the activity of the patient's disease than upon the dietary protein intake.

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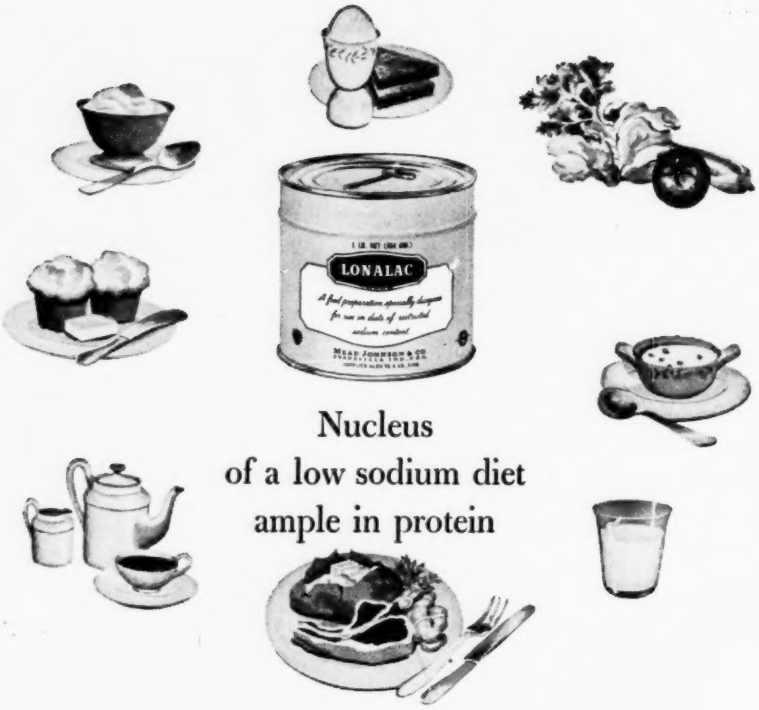

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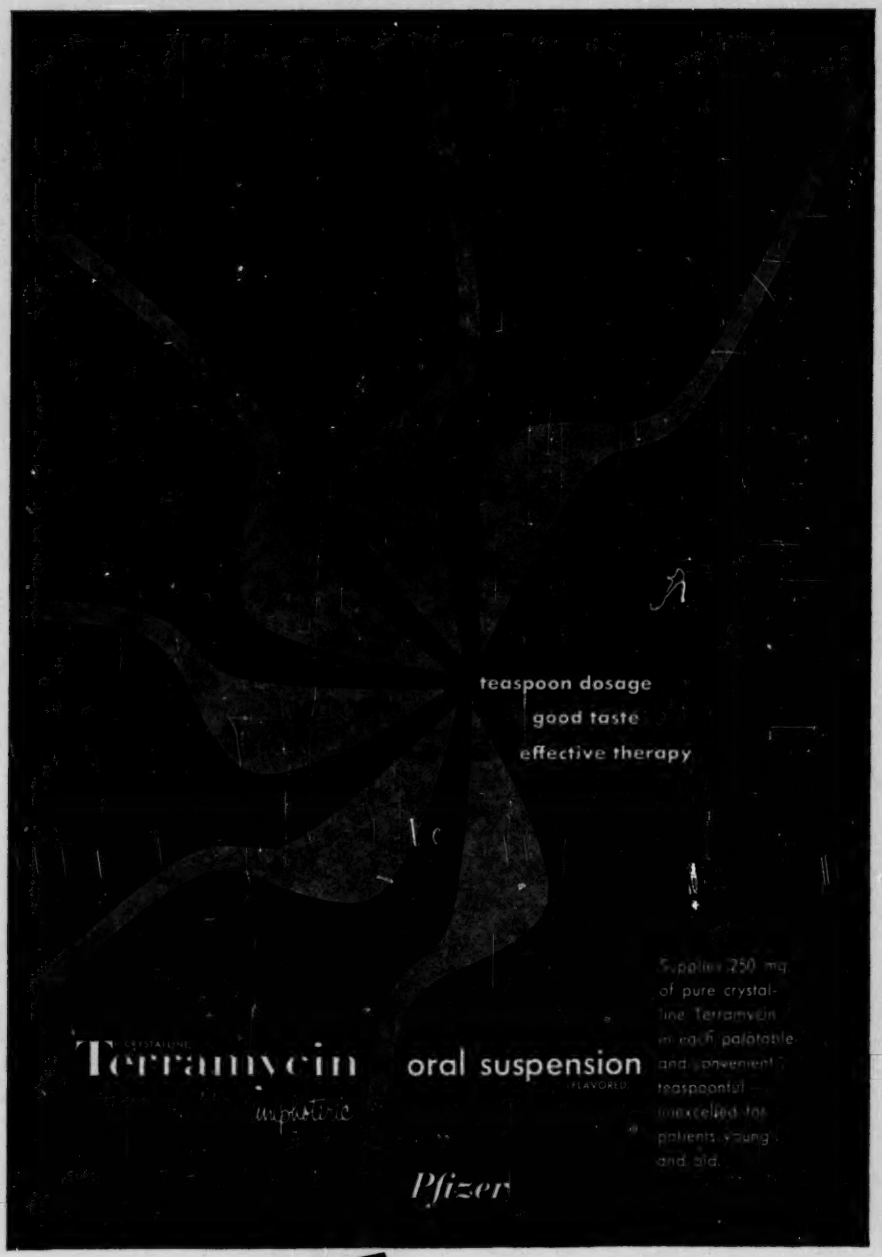

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